RESPONSE

I. Status of the Claims

No claims have been amended. New claim 9 has been added to better claim the present invention. Claims 1-3 were previously cancelled and claims 5-8 added. Thus claims 4-9 are presently pending in this case.

II. Support for New Claim

New claim 9 has been added to better claim the present invention. Claim 9 finds support throughout the specification, claims and sequence listing as originally filed and more specifically from original claim 4,

and SEQ ID NO:3 .

As new claim 9 is completely supported by the specification, claims and sequence listing as originally filed and contains no new matter, entry is respectfully requested.

III. Rejection of Claims Under 35 U.S.C. § 101

The Action rejects claims 4-8 under 35 U.S.C. § 101, because the claimed invention is not supported by either a substantial asserted utility or well-established utility. Applicants respectfully traverse.

The Action discounts many of the numerous utilities described in the specification for the sequences of the present invention based on the position that while credible, these utilities are not specific or substantial. While Applicants in no way agree with the Examiner's arguments, Applicants have chosen to expand on only a few of the utilities, as only one is required.

Applicants respectfully submit that the legal test for utility involves an assessment of whether those skilled in the art would find any of the utilities described for the invention to be credible or believable. According to the Examination Guidelines for the Utility Requirement, if the applicant has asserted that the claimed invention is useful for any particular purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, the Examiner should not impose a rejection based on lack of utility (66 Federal Register 1098, January 5, 2001).

In *In re Brana,* (34 USPQ2d 1436 (Fed. Cir. 1995), "*Brana*"), the Federal Circuit admonished the P.T.O. for confusing "the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption". *Brana* at 1442. The Federal Circuit went on to state:

> At issue in this case is an important question of the legal constraints on patent office examination practice and policy. The question is, with regard to pharmaceutical inventions, what must the applicant provide regarding the practical utility or usefulness of the invention for which patent protection is sought. This is not a new issue; it is one which we would have thought had been settled by case law years ago.

Brana at 1439, emphasis added. The choice of the phrase "utility or usefulness" in the foregoing quotation is highly pertinent. The Federal Circuit is evidently using "utility" to refer to rejections under 35 U.S.C. § 101, and is using "usefulness" to refer to rejections under 35 U.S.C. § 112, first paragraph. This is made evident in the continuing text in *Brana*, which explains the correlation between 35 U.S.C. §§ 101 and 112, first paragraph. The Federal Circuit concluded:

> FDA approval, however, is not a prerequisite for finding a compound useful within the meaning of the patent laws. Usefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans. Were we to require Phase II testing in order to prove utility, the associated costs would prevent many companies from obtaining patent protection on promising new inventions, thereby eliminating an

> incentive to pursue, through research and development, potential cures in many crucial areas such as the treatment of cancer.

Brana at 1442-1443, citations omitted. In assessing the question of whether undue experimentation would be required in order to practice the claimed invention, the key term is "undue", not "experimentation". *In re Angstadt and Griffin*, 190 USPQ 214 (C.C.P.A. 1976). The need for some experimentation does not render the claimed invention unpatentable. Indeed, a considerable amount of experimentation may be permissible if such experimentation is routinely practiced in the art. *In re Angstadt and Griffin, supra*; *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 18 USPQ2d 1016 (Fed. Cir. 1991). As a matter of law, it is well settled that a patent need not disclose what is well known in the art. *In re Wands*, 8 USPQ 2d 1400 (Fed. Cir. 1988).

Even under the newly installed utility guidelines, Applicants note that MPEP 2107 (II)(B)(1) states:

> (1) If the applicant has asserted that the claimed invention is useful for any particular practical purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, do not impose a rejection based on lack of utility. (MPEP 2107 (II)(B)(1))

Applicants have asserted that the sequences of the present invention encode a novel human dectin protein (for example, in the title of the application). Dectins are dendritic cell lectins. Lectins facilitate the binding of ligand and are known to play a roll in cell adhesion, cell-cell signalling, glycoprotein turnover, and (particularly so for dectins) a role in inflammation and immune response (specifically the innate immune response). The sequences of the presently claimed invention encode a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily. In support of this assertion Applicants invite the Examiners attention to **Exhibit A** which is a sequence alignment between SEQ ID NO: 3 and GENBANK accession number NM_203503 (summary of provided as **Exhibit**

B). NM_203503 is annotated as a transcript variant 2 of human C-type lectin domain family 4, member C (CLEC4). From the alignment provided in Exhibit A , one can see that the coding sequence provided in the SEQ ID NO:3 encodes a shorter isoform that is 99.818% identical with the transcript of NM_203503. Thus, it is clear that the sequences of the claimed invention encode a shorter isoform of a human dectin as was asserted in the present application as filed.

Additionally Applicants respectfully submit a series of scientific publications which were linked by NCBI to GENBANK accession number NM_203503 (publications listed in Exhibit B). The scientific publications by Dzionek, *et al.* , 2000 (J. Immunol. **165** (11), 6037-6046: **Exhibit C**) ; Fernandes, *et al.*, 2000 (Genomics **69** (2), 263-270: Abstract provided as **Exhibit D**); Dzionek, *et al.*, 2001 (J. Exp. Med. **194** (12), 1823-1834: **Exhibit E**); and Arce, *et al.*, 2001 (Eur. J. Immunol. 31 (9), 2733-2740: Abstract provided as **Exhibit F**) clearly indicate that those of skill in the art the specific utility of the claimed invention. These publications were contemporaneous with the filing of the instant application, which indicates that those of skill in the art recognized, at the time the instant application was filed, the specific utility of the claimed invention. Thus clearly the claimed sequences have specific patentable utility and this rejection should be withdrawn.

The legal test for utility simply involves an assessment of whether those skilled in the art would find any of the utilities described for the invention to be credible or believable. Given the clear recognition of those of skill in the art as described in the referred to publication, there can be no question that those skilled in the art would clearly believe that the molecule encoded by the sequences of the present invention have specific, substantial and well established and "real world" utility. As such, the scientific evidence provided clearly establishes that Applicants have described an invention whose

utility is in full compliance with the provisions of 35 U.S.C. § 101, and therefore Applicants respectfully request withdrawal of the rejection.

The question of utility is a straightforward one. As set forth by the Federal Circuit, "(t)he threshold of utility is not high: An invention is 'useful' under section 101 if it is capable of providing some identifiable benefit." *Juicy Whip Inc. v. Orange Bang Inc.*, 51 USPQ2d 1700 (Fed. Cir. 1999) (citing *Brenner v. Manson*, 383 U.S. 519, 534 (1966)). Additionally, the Federal Circuit has stated that "(t)o violate § 101 the claimed device must be totally incapable of achieving a useful result." *Brooktree Corp. v. Advanced Micro Devices, Inc.*, 977 F.2d 1555, 1571 (Fed. Cir. 1992), emphasis added. *Cross v. Iizuka* (224 USPQ 739 (Fed. Cir. 1985); "*Cross*") states "any utility of the claimed compounds is sufficient to satisfy 35 U.S.C. § 101". *Cross* at 748, emphasis added. Indeed, the Federal Circuit recently emphatically confirmed that "anything under the sun that is made by man" is patentable *(State Street Bank & Trust Co. v. Signature Financial Group Inc.*, 47 USPQ2d 1596, 1600 (Fed. Cir. 1998), citing the U.S. Supreme Court's decision in *Diamond vs. Chakrabarty*, 206 USPQ 193 (S.Ct. 1980)).

The legal test for utility simply involves an assessment of whether those skilled in the art would find any of the utilities described for the invention to be credible or believable. According to the Examination Guidelines for the Utility Requirement, if the applicant has asserted that the claimed invention is useful for any particular purpose (i.e., it has a "specific and substantial utility") and the assertion would be considered credible by a person of ordinary skill in the art, the Examiner should not impose a rejection based on lack of utility. Therefore , the present claims clearly meet the requirements of 35 U.S.C. § 101.

Although the above discussion is believed to be dispositive of the utility issue, Applicants would like to further direct the Examiner's attention to the parts of the specification that describe the use of sequences in a gene chip format to provide a high throughput analysis of the relevant cellular "transcriptome", including assessing temporal and tissue specific gene expression patterns, particularly using a high throughput "chip" format (specification at or about page 6, line 3 through page 9).

Evidence of the "real world" substantial utility of the present invention is further provided by the fact that there is an entire industry established based on the use of gene sequences or fragments thereof in a gene chip format. Perhaps the most notable gene chip company is Affymetrix. However, there are many companies which have, at one time or another, concentrated on the use of gene sequences or fragments, in gene chip and non-gene chip formats, for example: Gene Logic, ABI-Perkin-Elmer, HySeq and Incyte. In addition, one such company, Rosetta Inpharmatics, was viewed to have such "real world" value that it was acquired by large pharmaceutical company, Merck & Co., for substantial sums of money (net equity value of the transaction was $620 million). The "real world" substantial industrial utility of gene sequences or fragments would, therefore, appear to be widespread and well established. Clearly, persons of skill in the art, as well as venture capitalists and investors, readily recognize the utility, both scientific and commercial, of genomic data in general, and specifically human genomic data. Billions of dollars have been invested in the human genome project, resulting in useful genomic data (see, *e.g.*, Venter *et al.*, 2001, Science *291*:1304, presented as **Exhibit G**). The results have been a stunning success as the utility of human genomic data has been widely recognized as a great gift to humanity (see, *e.g.*, Jasny and Kennedy, 2001, Science *291*:1153, presented as **Exhibit H**). Clearly, the usefulness of human genomic data, such as the presently claimed nucleic acid molecules, is substantial and credible (worthy of billions of dollars and the creation of numerous companies focused

on such information) and well-established (the utility of human genomic information has been clearly understood for many years). The sequences of the present invention have particularly specific utility in DNA gene chip based analysis as they have been identified to contain several coding region single nucleotide polymorphisms (cSNPs), thus increasing their utility in DNA gene chip based analysis.

DNA chips clearly have utility, as evidenced by hundreds of issued U.S. Patents, as exemplified by U.S. Patent Nos. 5,445,934, 5,556,752, 5,744,305, 5,837,832, 6,156,501 and 6,261,776 (**Exhibits I-N**; copies of issued U.S. Patents not provided pursuant to current United States Patent and Trademark Office policy). Accordingly, the present sequence has a specific utility in such DNA chip applications. Clearly, compositions that enhance the utility of such DNA chips, like the present sequences, which encodes a novel human dectin proteins, that has been associated with may human porcesses, must have utility. The sequences of the present invention which encode a human dectin protein and thus provides a specific marker for the human genome (see also chromosome mapping discussion below and information provided in the specification at page 18, lines 6-7 that indicate that this protein is encoded on human chromosome 7). Thus, those skilled in the art would instantly recognize that the sequences of the present invention would be an ideal, novel candidate for assessing gene expression using, for example, DNA chips, as the specification details. Accordingly, the present sequence has a specific utility in such DNA chip applications. Clearly, compositions that enhance the utility of such DNA chips, such as the presently claimed nucleotide sequence encoding a novel human dectin protein, must also be useful.

The Examiner is further requested to consider that, given the huge expense of the drug discovery process, even negative information obtained using these specific markers of expression of a human dectin protein provides a very specific markers for the human genome and have great "real

world" practical utility. Knowing that a given gene is not expressed in medically relevant tissue provides an informative finding of great value to industry by allowing for the more efficient deployment of expensive drug discovery resources. Such practical considerations are equally applicable to the scientific community in general, in that time and resources are not wasted chasing what are essentially scientific dead-ends (from the perspective of medical relevance). Clearly, compositions that enhance the utility of DNA gene chips, such as the presently claimed sequences encoding a human dectin protein, must in themselves be useful. Moreover, the presently described human dectin protein sequences provide uniquely specific sequence resources for identifying and quantifying full length transcripts that were encoded by the corresponding human genomic locus. Accordingly, there can be no question that the described sequences provide an exquisitely specific utility for analyzing gene expression. Thus, the present claims clearly meet the requirements of 35 U.S.C. § 101.

Further evidence of utility of the presently claimed polynucleotide, although only one is needed to meet the requirements of 35 U.S.C. § 101 (*Raytheon v. Roper*, 220 USPQ 592 (Fed. Cir. 1983); *In re Gottlieb*, 140 USPQ 665 (CCPA 1964); *In re Malachowski*, 189 USPQ 432 (CCPA 1976); *Hoffman v. Klaus*, 9 USPQ2d 1657 (Bd. Pat. App. & Inter. 1988)), is the specific utility the present nucleotide sequence has in determining the genomic structure of the corresponding human chromosome, for example mapping the protein encoding regions as described in the specification. Clearly, the present polynucleotide provides exquisite specificity in localizing the specific region of the human chromosome 8 containing the gene encoding this protein, a utility not shared by virtually any other nucleic acid sequence. In fact, it is this specificity that makes this particular sequence so useful. Early gene mapping techniques relied on methods such as Giemsa staining to identify regions of chromosomes. However, such techniques produced genetic maps with a resolution of only 5 to 10 megabases, far too low to be

of much help in identifying specific genes involved in disease. The skilled artisan readily appreciates the significant benefit afforded by markers that map a specific locus of the human genome, such as the present nucleic acid sequence.

The Action discounts Applicant's assertion regarding the use of the presently claimed polynucleotides for gene mapping and determining chromosome structure again based on the position that such a use would allegedly be generic and therefore fail to represent a specific and substantial utility. However, as only a minor percentage of the genome actually encodes exons, which in turn encode amino acid sequences, the presently claimed polynucleotide sequence provides biologically validated empirical data (*e.g.*, showing which sequences are transcribed, spliced, and polyadenylated) that *specifically* defines that portion of the corresponding genomic locus that actually encodes exon sequence. Equally significant is that the claimed polynucleotide sequence defines how the encoded exons are actually spliced together to produce an active transcript (*i.e.*, the described sequences are useful for functionally defining exon splice-junctions). The Applicants respectfully submit that the practical scientific value of expressed, spliced, and polyadenylated mRNA sequences is readily apparent to those skilled in the relevant biological and biochemical arts. For further evidence supporting the Applicants' position, the Board is requested to review, for example, section 3 of Venter *et al.* (*supra at* pp. 1317-1321, including Fig. 11 at pp.1324-1325), which demonstrates the significance of expressed sequence information in the structural analysis of genomic data. The presently claimed polynucleotide sequence defines a biologically validated sequence that provides a unique and specific resource for mapping the genome essentially as described in the Venter *et al.* article.

In addition, among other things the mapping of the relatively few expressed human genes to a particular chromosome has long been a recognized method of identifying a genes associated with

particular diseases. Furthermore, the mapping of the human chromosome is a project of such widely recognized importance by those of skill in the art and even lay people, that both the US government and private corporations have dedicated millions of dollars to such a project. One is thus forced to ask, if the mapping of human chromosomes is a throw away utility then why has the US government spent so many taxpayer dollars on this project?

The Action's position is that this utility, like the use of these specific sequences on DNA chips or the described polymorphisms in forensic analysis, is that since other molecules can be used to map the human chromosome or on DNA chips or in forensic analysis, these utilities are not specific or substantial. As described previously above, Appellants once again point out that these arguments are completely rebuffed by the Federal Circuit's holding in *Carl Zeiss Stiftung v. Renishaw PLC*, 20 USPQ2d 1101 (Fed. Cir. 1991; "*Carl Zeiss*":"[A]n invention need not be the best or only way to accomplish a certain result").

Furthermore, the argument that just because there are other objects having the same utility, that utility has been rendered generic and therefore invalid begs the question, previously presented, that don't all golf balls and tires have the same utility of other golf balls or tires, i.e. they can be used as golf balls or tires respectively and yet these items are readily considered to have patentable utility.

It has been clearly established that a statement of utility in a specification must be accepted absent reasons why one skilled in the art would have reason to doubt the objective truth of such statement. *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA, 1974; "*Langer*"); *In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA, 1971). As clearly set forth in *Langer*:

> As a matter of Patent Office practice, a specification which contains a disclosure of utility which corresponds in scope to the subject matter sought to be patented must be

> taken as sufficient to satisfy the utility requirement of § 101 for the entire claimed subject matter unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope.

Langer at 297, emphasis in original. As set forth in the MPEP, "Office personnel must provide evidence sufficient to show that the statement of asserted utility would be considered 'false' by a person of ordinary skill in the art" (MPEP, Eighth Edition at 2100-40, emphasis added). Thus, the present claims clearly meet the requirements of 35 U.S.C. § 101.

Finally, with full recognition of the fact that all patent applications are examined on their own merits and that the prosecution of one patent does not effect the prosecution of another patent, *In re Wertheim,* 541 F.2d 257, 264, 191 USPQ 90, 97 (CCPA 1976), however the issue at hand in one of whether the fact that patents have issued recognizing the utility of a class of molecules does this confers a statutory precedent of patentability to a broad class of compositions. Thus, there remains a lingering issue regarding due process and equitable treatment under the law. While Applicants are well aware of the new Utility Guidelines set forth by the USPTO, Applicants respectfully point out that the current rules and regulations regarding the examination of patent applications is and always has been the patent laws as set forth in 35 U.S.C. and the patent rules as set forth in 37 C.F.R., not the Manual of Patent Examination Procedure or particular guidelines for patent examination set forth by the USPTO. Furthermore, it is the job of the judiciary, not the USPTO, to interpret these laws and rules. Applicants are unaware of any significant recent changes in either 35 U.S.C. § 101, or in the interpretation of 35 U.S.C. § 101 by the Supreme Court or the Federal Circuit that is in keeping with the new Utility Guidelines set forth by the USPTO. This is underscored by numerous patents that have been issued over the years that claim nucleic acid fragments that do not comply with the new Utility Guidelines. As examples of such issued U.S. Patents, the Examiner is invited to review U.S. Patent

Nos. 5,817,479, 5,654,173, and 5,552,281 (each of which claims short polynucleotides; **Exhibits O-Q**; copies of issued U.S. Patents not provided pursuant to current United States Patent and Trademark Office policy), and recently issued U.S. Patent No. 6,340,583 (which includes no working examples; **Exhibit R**; copies of issued U.S. Patents not provided pursuant to current United States Patent and Trademark Office policy), none of which contain examples of the "real-world" utilities that the Examiner appears to desire. As issued U.S. Patents are presumed to meet all of the requirements for patentability, including 35 U.S.C. §§ 101 and 112, first paragraph (see Section IV, below), Applicants submit that the present polynucleotides must also meet the requirements of 35 U.S.C. § 101. While Applicants agree that each application is examined on its own merits, Applicants are unaware of any changes to 35 U.S.C. § 101, or in the interpretation of 35 U.S.C. § 101 by the Supreme Court or the Federal Circuit, since the issuance of these patents that render the subject matter claimed in these patents, which is similar to the subject matter in question in the present application, as suddenly non-statutory or failing to meet the requirements of 35 U.S.C. § 101. Thus, holding Appellants invention to a different standard of utility appears inconsistent and inequitable, such a judgement being arbitrary and capricious, a violation of due process and equal protection under the law and cannot be maintained.

In light of the evidence presented herewith and for the many compelling reasons described above, it is clear that the present invention clearly has utilities that are specific, substantial and credible. Therefore, Applicants submit that the rejection of the pending claims under 35 U.S.C. § 101 has been avoided and respectfully request withdrawal of the pending rejection of claims under 35 U.S.C. § 101.

IV. Rejection of Claims Under 35 U.S.C. § 112, First Paragraph

The Action next rejects claims 4-8 under 35 U.S.C. § 112, first paragraph, since allegedly one skilled in the art would not know how to use the invention, as the invention allegedly is not supported

by a specific, substantial, and credible utility or a well-established utility. Applicants respectfully traverse.

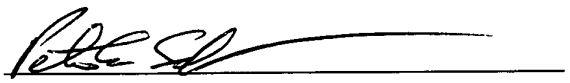
Applicants submit that as claims 4-8 have been shown by contemporaneous scientific publication to have a recognized "a specific, substantial, and credible utility", as detailed in section III above, the present rejection of claims 4-8 under 35 U.S.C. § 112, first paragraph, cannot stand.

Applicants therefore request that the rejection of the pending claims under 35 U.S.C. § 112, first paragraph, be withdrawn.

V. Conclusion

The present document is a full and complete response to the Action. In conclusion, Applicants submit that, in light of the foregoing amendments and remarks, the present case is in condition for allowance, and such favorable action is respectfully requested. Should Examiner Pak have any questions or comments, or believe that certain amendments of the claims might serve to improve their clarity, a telephone call to the undersigned Applicants' representative is earnestly solicited.

Respectfully submitted,



November 29, 2005
Date

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Customer # 24231

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# /usr/local/lexicon/bin/fasta34 -Q /tmp/fasta3.SjayEk /tmp/fasta3.TjayEk
FASTA searches a protein or DNA sequence data bank
 version 3.4t25 Nov 12, 2004
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

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             470       480       490       500       510       520

              40        50        60        70        80        90
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             160       170       180       190       200       210
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             220       230       240       250       260       270
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gi|455 CAATCTTGGACTAAGAGTCAAAAGAACTGTTCTGTGATGGGGGCTGATCTGGTGGTGATC
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             280       290       300       310       320       330
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       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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             770       780       790       800       810       820

              340       350       360       370       380       390
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       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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              400       410       420       430       440       450
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       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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             890       900       910       920       930       940

              460       470       480       490       500       510
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       ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
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             950       960       970       980       990      1000

              520       530       540
hMEM_3 CAGAAGTCAATTTGCAAGATGAAGAAGATCTACATATAA
       ::::::::::::::::::::::::::::::::::::
gi|455 CAGAAGTCAATTTGCAAGATGAAGAAGATCTACATATAAATGAAATATTCTCCCTGGAAA
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     720       730       740       750       760       770

              240       230       220       210       200       190
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REFERENCE   1  (bases 1 to 1221)
  AUTHORS   Clark,H.F., Gurney,A.L., Abaya,E., Baker,K., Baldwin,D., Brush,J.,
            Chen,J., Chow,B., Chui,C., Crowley,C., Currell,B., Deuel,B.,
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            Heldens,S., Huang,A., Kim,H.S., Klimowski,L., Jin,Y., Johnson,S.,
            Lee,J., Lewis,L., Liao,D., Mark,M., Robbie,E., Sanchez,C.,
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TITLE BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood
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AUTHORS Fernandes,M.J., Iscove,N.N., Gingras,G. and Calabretta,B.
TITLE Identification and characterization of the gene for a novel C-type lectin (CLECSF7) that maps near the natural killer gene complex on human chromosome 12
JOURNAL Genomics 69 (2), 263-270 (2000)
PUBMED 11031109
COMMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence was derived from AF293615.1.

Summary: This gene encodes a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily. Members of this family share a common protein fold and have diverse functions, such as cell adhesion, cell-cell signalling, glycoprotein turnover, and roles in inflammation and immune response. The encoded type 2 transmembrane protein may play a role in dendritic cell function. Two transcript variants encoding distinct isoforms have been identified for this gene.

Transcript Variant: This variant (2), also known as DLEC-beta, lacks an in-frame segment of the coding region, compared to variant 1. It encodes a shorter isoform (2), compared to isoform 1.
COMPLETENESS: complete on the 3' end.

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Nov 21 2005 12:16:20

BDCA-2, BDCA-3, and BDCA-4: Three Markers for Distinct Subsets of Dendritic Cells in Human Peripheral Blood

Andrzej Dzionek,* Anja Fuchs,* Petra Schmidt,* Sabine Cremer,* Monika Zysk,* Stefan Miltenyi,* David W. Buck,† and Jürgen Schmitz[1]*

We have generated a panel of mAbs that identify three presumably novel human dendritic cell Ags: BDCA-2, BDCA-3, and BDCA-4. In blood, BDCA-2 and BDCA-4 are expressed on CD11c$^-$ CD123^{bright} plasmacytoid dendritic cells, whereas BDCA-3 is expressed on small population of CD11c$^+$ CD123$^-$ dendritic cells. All three Ags are not detectable on a third blood dendritic cell population, which is CD1c$^+$ CD11c^{bright} CD123^{dim}, or on any other cells in blood. BDCA-4 is also expressed on monocyte-derived and CD34$^+$ cell-derived dendritic cells. Expression of all three Ags dramatically changes once blood dendritic cells undergo in vitro maturation. BDCA-2 is completely down-regulated on plasmacytoid CD11c$^-$ CD123^{bright} dendritic cells, expression of BDCA-3 is up-regulated on both plasmacytoid CD11c$^-$ CD123^{bright} dendritic cells and CD1c$^+$ CD11c^{bright} CD123^{dim} dendritic cells, and expression of BDCA-4 is up-regulated on CD1c$^+$ CD11c^{bright} CD123^{dim} dendritic cells. BDCA-2 is rapidly internalized at 37°C after mAb labeling. The three presumably novel Ags serve as specific markers for the respective subpopulations of blood dendritic cells in fresh blood and will be of great value for their further analysis and to evaluate their therapeutic potential. *The Journal of Immunology,* **2000, 165: 6037–6046.**

Studies of blood dendritic cells (BDC)[2] have been hampered by their scarcity and the lack of BDC-specific cell surface markers. Methods for the detection and isolation of fresh noncultured BDC are commonly based on a multitude of immunophenotypic criteria, such as the absence of a panel of leukocyte lineage (lin)-specific Ags (e.g., CD3, CD14, CD19, and CD56) and the presence of HLA-DR, CD4 or CD33 (1–13).

From analyses of freshly isolated lin$^-$ BDC it became evident that BDC do not represent a homogeneous cell population, but, rather, a mixture of at least two populations (3–8, 10, 14): 1) CD123^{bright} CD11c$^-$ BDC, which possess a plasmacytoid morphology, express CD45RA, and depend on IL-3 for their survival and differentiation into mature DC with typical dendritic morphology and potent T cell stimulatory function; and 2) CD123^{dim} CD11c^{bright} BDC, which are rather monocytoid in appearance, express CD45RO, and spontaneously develop into typical mature DC even when cultured without any exogenous cytokines. Plasmacytoid CD123^{bright} CD11c$^-$ BDC display some features, such as the expression of the pre-TCR α-chain, that indicate that they may arise from lymphoid precursors (4, 14, 15), whereas CD123^{dim} CD11c^{bright} BDC display all the criteria of myeloid DC (3–7). DC resembling plasmacytoid CD123^{bright} CD11c$^-$ BDC have been detected in the T cell-rich areas of lymphoid tissue (16) and were initially erroneously designated plasmacytoid T cells or plasmacytoid monocytes due to their morphology and phenotype (17, 18). DC resembling CD123^{dim} CD11c^{bright} BDC have been found in the dark and light zones of germinal centers (19).

Here we describe a panel of new mAb raised against immunomagnetically purified CD4$^+$ lin$^-$ BDC that identify three presumably novel BDC Ags: BDCA-2, BDCA-3, and BDCA-4. In fresh human blood, expression of BDCA-2 and BDCA-4 is strictly confined to plasmacytoid CD123^{bright} CD11c$^-$ BDC, whereas expression of BDCA-3 is restricted to a small population of CD123$^-$ CD11c$^+$ BDC. This small population of BDCA-3$^+$ BDC shares many immunophenotypic features with classical CD123^{dim} CD11c^{bright} BDC, but unlike CD123^{dim} CD11c^{bright} BDC, BDCA-3$^+$ BDC lack expression of CD1c (BDCA-1), CD2, and several of the Fc receptors.

Materials and Methods

Generation of BDC-specific mAb

Five 6- to 8-wk-old female BALB/c mice (Simonsen, Gilroy, CA) were inoculated with ~5 × 10^5 to 1 × 10^6 purified HLA-DR$^+$ lin$^-$ BDC under anesthesia on days 0, 4, 7, 11, and 14 in the right hind footpad and with ~1 × 10^6 HLA-A2$^+$ Bristol-8 B lymphoblastoma cells in the left hind footpad on days −3, 0, 4, 7, 11, and 14 (30). Both cell types were incubated with 1/100 PHA (Life Technologies, Gaithersburg, MD) for 10 min at room temperature and washed with PBS before injection.

On day 15 the cells of the mouse right hind popliteal lymph nodes were fused to SP2/0 Ag14 myeloma cells. Fused cells were plated on 96-well plates in DMEM supplemented with 20% FCS (HyClone, Logan, UT), 2 mmol/L L-glutamine, 15 mmol/L HEPES, 10^{-4} mmol/L hypoxanthine (Life Technologies), and 2 μg/ml azaserine (*O*-diazoacetyl-L-serine; Sigma, St. Louis, MO) and placed in a 37°C incubator with 9% CO_2.

When visible hybridoma colonies were apparent, flow cytometric analysis was used to screen supernatants from these wells for Ab secretion and for nonreactivity (≤1% positive cells) to PBMC. Briefly, a mixture of rat anti-mouse κ mAb-conjugated polystyrene beads (2.5 μm in diameter; Interfacial Dynamics, Portland, OR) and PBMC was incubated with 50 μl of hybridoma supernatant for 20 min at room temperature. The bead/cell mixture was then washed twice with PBS, pH 7.4, containing 5 mmol/L EDTA and 0.5% BSA (PBS/EDTA/BSA), and binding of mouse IgM, IgG1, Ig2a, and Ig2b from the supernatants to the beads and the test cells was detected by staining with PE-conjugated rat anti-mouse IgM mAb (clone X54; BD Biosciences, San Jose, CA), rat anti-mouse IgG1 mAb (clone X56; BD Biosciences), and rat anti-mouse IgG2 mAb (clone X57; BD Biosciences).

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[2] Abbreviations used in this paper: BDC, blood dendritic cell; DC, dendritic cell; lin, lineage; MACS, magnetic cell sorting; Mo-DC, monocyte-derived DC; CD34-derived DC, CD34$^+$ hemopoietic progenitor cell-derived DC; LY, Lucifer Yellow.

0022-1767/00/$02.00

Culture supernatants that fulfilled the screening criteria of the first round were then flow cytometrically screened for reactivity to a significant proportion of BDC. Briefly, a mixture of rat anti-mouse κ mAb-conjugated polystyrene beads and enriched BDC (PBMC depleted of B cells, T cells, and monocytes) was incubated with 50 μl of hybridoma culture supernatant for 20 min at room temperature. The mixture was then washed twice with PBS/EDTA/BSA and stained with PE-conjugated rat anti-mouse IgM mAb, rat anti-mouse IgG1 mAb, and rat anti-mouse IgG2 mAb to detect binding of mouse IgM, IgG1, Ig2a, and Ig2b from the supernatants to the beads and the enriched BDC. For discrimination of HLA-DR$^+$ BDC from HLA-DR$^-$ cells, the bead/cell mixture was then washed once, free binding sites of the PE-conjugated rat anti-mouse IgG2 mAb and the bead-conjugated rat anti-mouse κ mAb were saturated by incubation with 100 μg/ml mouse IgG2a, and the mixture was counterstained with anti HLA-DR-FITC (clone AC122, IgG2a).

Selected hybridoma cells were expanded, subclones were established, and the isotype of the mAb was determined by the ISOTYPE Ab-STAT Kit (SangStat Medical, Palo Alto, CA).

Cell preparations

Buffy coats from normal healthy volunteers were obtained form the Institute for Transfusionmedicine, Hospital Merheim (Cologne, Germany). PBMC were prepared from buffy coats by standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Peripheral blood leukocytes were prepared from buffy coats by lysis of erythrocytes in isotonic ammonium chloride buffer.

CD4$^+$ lin$^-$ BDC were isolated from PBMC by two-step immunomagnetic cell sorting (MACS) as described in detail previously (13, 20). Briefly, monocytes, T cells, B cells, and NK cells were depleted using mAb against CD3 (clone BW264/56), CD11b (clone M1/70.15.11.5), CD16 (clone VEP-13), and in a few experiments a poorly defined Ag expressed on B cells and monocytes (clone L179; BD Biosciences). From the depleted cell fraction, BDC were then enriched using an mAb against CD4 (clone M-T321). To screen hybridoma culture supernatants (see above), BDC were merely partially enriched by immunomagnetic depletion of T cells, B cells, and monocytes based on CD3 and L179 Ag expression.

CD1c-, BDCA-2-, and BDCA-3-expressing cells were isolated from PBMC by indirect magnetic labeling with PE- or FITC-conjugated mAb (AD5-8E7, AC144, and AD5-5E8, respectively) and anti-PE or anti-FITC mAb-conjugated microbeads (Miltenyi Biotec) and enrichment of labeled cells by MACS. In some experiments BDCA-3$^+$ cells were isolated based on direct magnetic labeling with anti-BDCA-3 mAb (AD5-5E8)-conjugated microbeads. Highly pure CD1c$^+$ BDC without contaminating CD1c$^+$ B cells were obtained by immunomagnetic depletion of CD19$^+$ B cells using CD19 mAb-conjugated microbeads (Miltenyi Biotec) followed by immunomagnetic enrichment of CD1c$^+$ cells.

Basophils were purified from PBMC by immunomagnetic depletion of nonbasophils based on indirect magnetic labeling of CD3-, CD7-, CD14-, CD15-, CD36-, CD45RA-, and HLA-DR-expressing cells with a magnetic labeling kit (Miltenyi Biotec). CD14$^+$ monocytes, CD34$^+$ hemopoietic progenitor cells, and CD3$^+$ T cells were immunomagnetically purified based on direct magnetic labeling with CD14, CD34, and CD3 mAb-conjugated microbeads (Miltenyi Biotec), respectively.

Cell culturing

For generation of immature monocyte-derived DC (Mo-DC), purified CD14$^+$ monocytes were cultured at a cell density of 5×10^5 to 1×10^6 cells/ml in medium (RPMI 1640 (Life Technologies) supplemented with 2 mmol/L L-glutamine, 10% FCS (Sigma), 110 mg/L sodium pyruvate (Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies)) at 37°C in a humidified 5% CO_2-containing atmosphere in the presence of 500-1000 U/ml rIL-4 (PeproTech, Rocky Hill, NJ) and 100 ng/ml rGM-CSF (PeproTech) for 7 days. For generation of mature Mo-DC, immature Mo-DC were washed once and cultured in medium in the presence of 20 ng/ml TNF-α (PeproTech) for another 3 days. For generation of CD34$^+$ hemopoietic progenitor cell-derived DC (CD34-derived DC), purified CD34$^+$ cells were cultured at a cell density of 5×10^4 cells/ml in medium in the presence of 100 ng/ml rFlt3 ligand (PeproTech), 0.5 ng/ml rTGF-β1 (PeproTech), 10 ng/ml rTNF-α, 20 ng/ml recombinant stem cell factor (PeproTech), and 100 ng/ml rGM-CSF for 11 days. Freshly isolated CD4$^+$ lin$^-$ BDC were cultured at a cell density of 5×10^5 to 1×10^6 cells/ml in medium in the presence of 10 ng/ml rIL-3 (PeproTech) for up to 48 h. Isolated CD1c-, BDCA-2-, and BDCA-3-expressing BDC were cultured at a cell density of 5×10^5 to 1×10^6 cells/ml in medium without any cytokines or in the presence of 10 ng/ml rIL-3, 20 ng/ml IL-4 (PeproTech), and 100 ng/ml GM-CSF for up to 48 h.

Flow cytometric analysis

A FACScalibur (BD Biosciences) was used for one-, two-, three-, or four-color flow cytometry. Data for 5×10^3 to 2×10^5 cells/sample were acquired in list mode and analyzed using CellQuest software (BD Biosciences).

The following mAb (clone names) were used in this study for flow cytometry: CD1a (HI149), CD10 (HI10a), CD11a (G43-25B), CD11c (B-ly6), CD25 (M-A261), CD27 (M-T271), CD32 (FL18.26), CD38 (HIT2), CD40 (5C3), CD43 (1G10), CD54 (HA58), CD62L (Dreg 56), CD64 (10.1), CD69 (FN50), CD98 (HIM6), anti-HLA-DQ (Tü169), and anti-TCRαβ (T10B9.1A-31) from PharMingen (San Diego, CA); CD2 (S5.2), CD8 (SK1), CD13 (L138), CD14 (MFP9), CD19 (SJ25-C1), CD33 (P67.6), CD34 (8G12), CD45RO (UCHL-1), CD56 (NCAM16.2), CD62L (SK11), CD71 (L01.1), CD123 (9F5), anti-IgD (TA4.1), anti-mouse IgG1 (X56), anti-mouse IgG2 (X57), and anti-mouse IgM (X54) from BD Biosciences; CD5 (CLB-T11/1, 6G4), CD7 (CLB-T-3A1/1, 7F3), CD16 (CLB-FcR Gran1, 5D2), CD45RA (F8-11-13), and CD80 (CLB-DAL1) from CLB (Amsterdam, The Netherlands); CD18 (7E4), CD23 (9P25), CD58 (AICD58), CD77 (38.13), CD83 (HB15A), CD86 (HA5.2B7), and CD116 (SC06) from Coulter-Immunotech (Marseilles, France); CD3 (BW264/56), CD4 (M-T321), CD11b (M1/70.15.11.5), CD14 (TUK4), CD15 (VIMC6), CD16 (VEP-13), anti-HLA-DR (910/D7), anti-AC133 (AC133/1), and anti-TCRαβ (BW242/412) from Miltenyi Biotec; CD36 (AC106), CD123 (AC145), anti-HLA-DR (AC122 and AC123), and anti-glycophorin A (AC107) from Amcell (Sunnyvale, CA); CD1c (M241) from Ancell (Bayport, MN); polyclonal anti-IgG, anti-IgM (SA-DA4), polyclonal anti-κ, and polyclonal anti-λ from Southern Biotechnology Associates (Birmingham, AL); CD61 (VIPL2) from W. Knapp (Institute of Immunology, University of Vienna, Vienna, Austria); CD44 (IM7) from J. Moll (Forschungszentrum Karlsruhe, Karlsruhe, Germany); CD20 (HI47) from Caltag (Burlingame, CA); anti-CLA (HECA-452) from E. Butcher (Department of Pathology, Stanford University, Stanford, CA); anti-FcεRI (15-1) from J. P. Kinet (Molecular Allergy and Immunology Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD); CD11c (Ki-M1) from M. R. Parwaresch (Department of Pathology, Christian Albrechts University, Kiel, Germany); CMRF-44 and CMRF-56 from D. N. Hart (Mater Medical Research Institute, Mater Misericordiae Hospitals, South Brisbane, Australia); and anti HLA-A,B,C (W6/32) from Sigma.

All Abs were used as FITC-, PE-, biotin-, or Cy5-conjugated mAb. For indirect immunofluorescent staining with biotinylated mAb, streptavidin-APC (BD Biosciences) was used. For exclusion of dead cells in the flow cytomeric analysis, cells were stained with propidium iodide. To minimize FcR-mediated mAb binding, cells were stained in most experiments in the presence of FcR-blocking reagent (Miltenyi Biotec) containing human IgG.

Microscopic analysis

Cells were spun down on slides in a cytocentrifuge (Cytospin 3; Shandon, Pittsburgh, PA). A Zeiss Axioscope microscope (Zeiss, Oberkochen, Germany) was used for analysis. Digital pictures were made using the Xillix MicroImager MI1400-12X (Xillix, Vancouver, Canada).

Endocytosis assay

To assess endocytosis of BDC subsets, purified CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC, and (as controls) purified CD3$^+$ T cells and immature Mo-DC were incubated at 37°C in medium with 1 mg/ml Lucifer Yellow (LY) for 0, 15, 45, and 75 min. Afterward, cells were washed three times in ice-cold PBS/EDTA/BSA and analyzed by flow cytometry.

Results

Reactivity with noncultured blood cells

The mAb listed in Table I fulfilled the initial screening criteria for BDC-specific mAb (see *Materials and Methods*) and were further characterized. According to their reactivity with blood cells, the mAb could be divided into four groups: 1) AC144, AD5-13A11, and AD5-4B8; 2) AD5-17F6; 3) AD5-5E8 and AD5-14H12; and 4) AD5-8E7.

The mAb of the first group, AC144, AD5-13A11, and AD5-4B8, stain 0.41 ± 0.17% (n = 10) of all PBMC (Fig. 1*A*). In a dot

Table I. *Monoclonal Ab with specificity for BDC*

Ag	Ab	Epitope	Isotype	$CD11c^{bright}$ $CD123^{low}$ BDC	$CD11c^{+}$ $CD123^{-}$ BDC	$CD11c^{-}$ $CD123^{bright}$ BDC	Other Leukocytes
BDCA-2	AC144	2A	IgG1	–	–	+	–
BDCA-2	AD5-13A11	2A	IgG2a	–	–	+	–
BDCA-2	AD5-4B8	2A	IgG1	–	–	+	–
BDCA-4	AD5-17F6	4A	IgG1	–	–	+	–
BDCA-3	AD5-5E8	3A	IgG1	–	+	–	–
BDCA-3	AD5-14H12	3B	IgG1	–	+	–	–
CD1c	AD5-8E7	1A	IgG2a	+	–	–	B cell subset

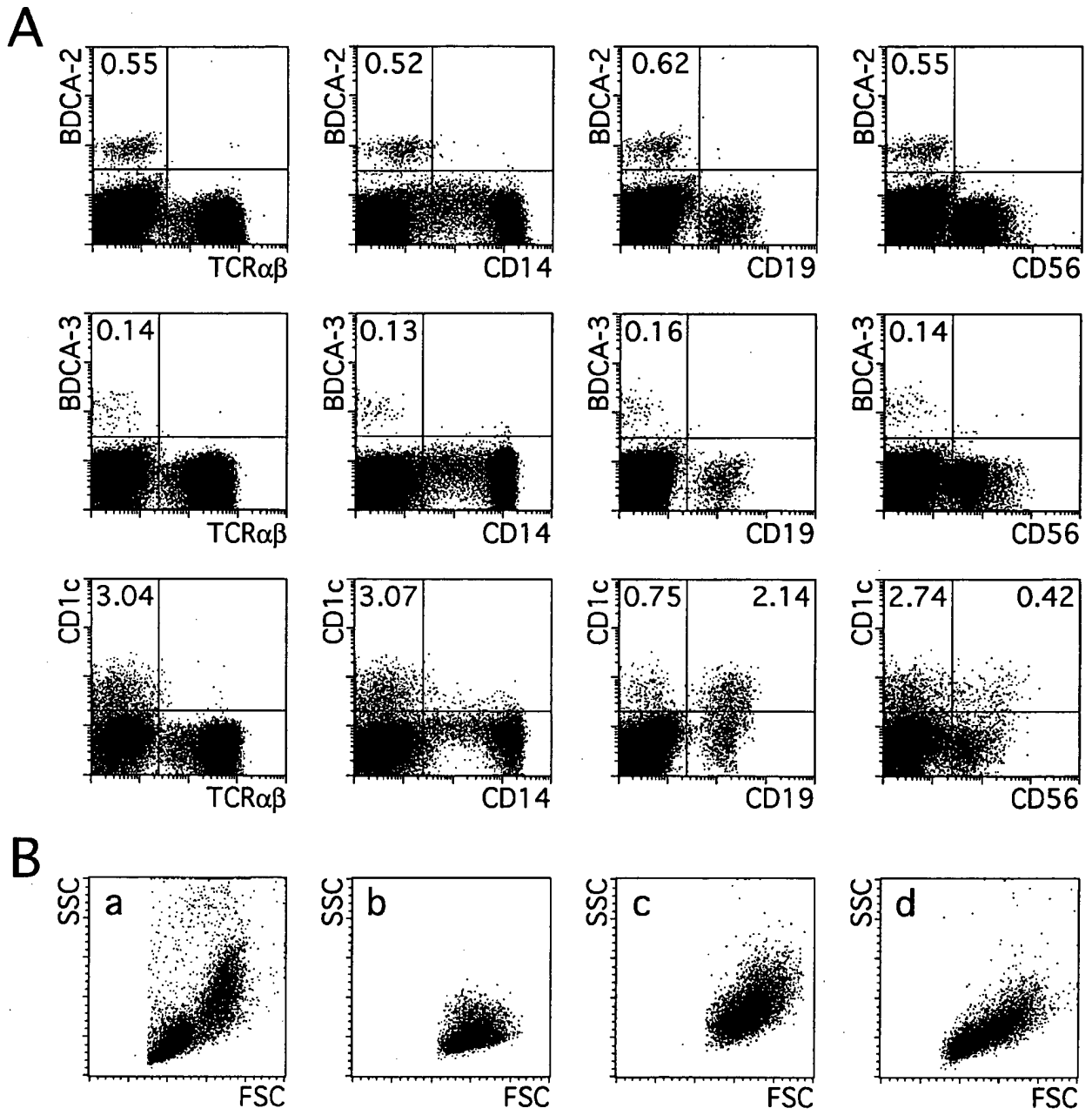

FIGURE 1. Expression of BDCA-2, BDCA-3, and CD1c (BDCA-1) on PBMC. *A*, Staining of PBMC with FITC-conjugated mAb against BDCA-2 (AC144), BDCA-3 (AD5-5E8), and CD1c (AD5-8E7) and with PE-conjugated mAb against the TCR$\alpha\beta$ heterodimer, CD14, CD19, and CD56, respectively. The numbers indicate the percentage of cells in the respective quadrant. Propidium iodide fluorescence and light scatter signals were used for gating of live cells. *B*, Scatter profile of PBMC (*a*), gated BDCA-2^{+} cells (*b*), gated BDCA-3^{+} cells (*c*), and gated $CD1c^{+}$ cells (*d*).

plot of forward and side scatter signals, these rare cells constitute a homogeneous cell population that is located between small resting lymphocytes and monocytes (Fig. 1*B*). Accordingly, these rare cells do not express TCR$\alpha\beta$, CD14, CD19, and CD56 (Fig. 1*A*), lineage markers that are expressed on T cells, monocytes, B cells, and NK cells, respectively. Staining of highly purified BDC (>95% HLA-DR$^+$, TCR$\alpha\beta^-$, CD14$^-$, CD19$^-$, and CD56$^-$) with CD123-PE vs CD11c-FITC reveals three BDC subsets (Fig. 2): 1) CD11c$^-$ CD123^{bright} BDC, 2) CD11c^{bright} CD123^{dim} BDC, and 3) CD11c$^+$ CD123$^-$ BDC. The mAb of the first group clearly stained all CD11c$^-$ CD123^{bright} BDC, but, except for a very few cells, not the CD11c$^+$ BDC (Fig. 2). To analyze whether all mAb of the first group react with a single Ag, we performed two-color stainings and cross-inhibition studies (data not shown). The results showed that all mAb of this group recognize a single epitope of the same Ag. We named this Ag BDCA-2.

As shown in Fig. 3, the mAb of the second group, AD5-17F6, recognizes the same cells among PBMC as AC144, one of the BDCA-2-specific mAb of the first group. Nevertheless, AD5-17F6 stains an Ag that is different from BDCA-2. This was unequivocally demonstrated by cointernalization experiments, where AD5-17F6 showed surface staining with equal intensity before and after anti-BDCA-2 mAb-mediated internalization of BDCA-2 (data not shown), and by staining of BDC after culture, where AC144 mAb and AD5-17F6 mAb showed entirely different staining patterns (see below and Fig. 4). The Ag recognized by AD5-17F6 was named BDCA-4.

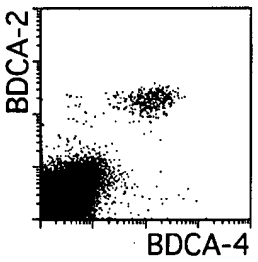

FIGURE 3. Expression of BDCA-4 on PBMC. Shown is a two-color staining of PBMC with FITC-conjugated mAb against BDCA-2 (AC144) and with PE-conjugated mAb against BDCA-4 (AD5-17F6). Note that almost no single-positive (BDCA-2$^+$ BDCA-4$^-$ and BDCA-2$^-$ BDCA-4$^+$) PBMC are detected.

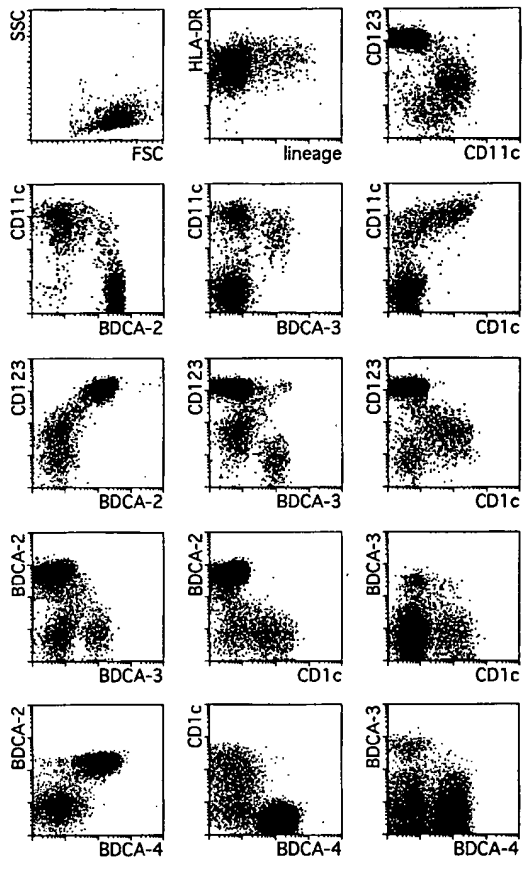

FIGURE 2. BDCA-2, BDCA-3, BDCA-4, and CD1c (BDCA-1) are expressed on three distinct subsets of BDC. BDC were isolated from PBMC by depletion of CD3-, CD11b-, and CD16-positive cells followed by enrichment of CD4-positive cells. The purity of BDC is demonstrated by light scatter properties (*upper left* dot plot) and anti-HLA-DR-Cy5 vs anti lin-FITC (anti-TCR$\alpha\beta$, CD14, CD19, and CD56) staining (*upper middle* dot plot). Note that only a few lin$^+$ cells are present. Expression of BDCA-2, BDCA-3, BDCA-4, and CD1c on BDC is characterized in a series of two-color stainings with PE- and FITC-conjugated mAb against CD11c, CD123, and the Ags themselves. Note that BDCA-2, BDCA-3, BDCA-4, and CD1c are almost exclusively expressed on only one of three distinct BDC subsets each. The subsets are defined according to staining of BDC with CD123-PE vs CD11c-FITC (*upper right* dot plot): 1) CD11c$^-$ CD123^{bright} BDC, 2) CD11c^{bright} CD123^{dim} BDC, and 3) CD11c$^+$ CD123$^-$ BDC.

The mAb of the third group, AD5-5E8 and AD5-14H12, stained 0.04 ± 0.01% (n = 10) of all PBMC (Fig. 1*A*). According to scatter signals (Fig. 1*B*) and counterstaining with mAb against TCR$\alpha\beta$, CD14, CD19, and CD56 (Fig. 1*A*), these cells are distinct from lymphocytes and monocytes and are slightly larger than the cells recognized by the Abs of the first group. Accordingly, staining of BDC shows that a different subset is recognized by AD5-5E8 and AD5-14H12, namely CD11c$^+$ CD123$^-$ BDC (Fig. 2). According to two-color stainings, cross-blocking studies and co-capping experiments (data not shown), both mAb appear to recognize two spatially unrelated epitopes of the same Ag. We named this Ag BDCA-3.

The fourth group mAb AD5-8E7 reacts with 2.39 ± 0.76% (n = 10) of unfractionated PBMC (Fig. 1*A*). Light scatter analysis (Fig. 1*B*) and counterstaining of the lineage markers TCR$\alpha\beta$, CD14, and CD19 revealed that the mAb is not reactive to T cells and monocytes, but is reactive to a major subset of small resting CD19$^+$ B cells. Staining of purified BDC shows that AD5-8E7, in addition to B cells, stains a third subset of BDC distinct from those subsets recognized by the mAb of the first and second groups, namely CD11c^{bright} CD123^{dim} BDC. A significant proportion of the CD11c^{bright} CD123^{dim} BDC expresses CD56 (see below). For this reason, some AD5-8E7-reactive PBMC stain for CD56 (Fig. 1*A*). AD5-8E7 is not reactive to purified NK cells (data not shown). The Ag recognized by AD5-8E7 was initially named BDCA-1, as it appeared to be a new Ag. However, it later transpired that AD5-8E7 completely blocked binding of the CD1c mAb M241 to MOLT-4 cells (Fig. 5). Thus, the Ag recognized by AD5-8E7 is CD1c.

None of the mAb listed in Table I is reactive to granulocytes, platelets, erythrocytes, purified basophils, or purified CD34$^+$ hemopoietic progenitor cells (data not shown).

Expression of BDCA-2, BDCA-3, and BDCA-4 on cultured BDC

Expression of BDCA-2, BDCA-3, and BDCA-4 on CD11c$^-$ and CD11c$^+$ BDC was analyzed after various periods of culturing total BDC in the presence of rIL-3. The results are shown in Fig. 4.

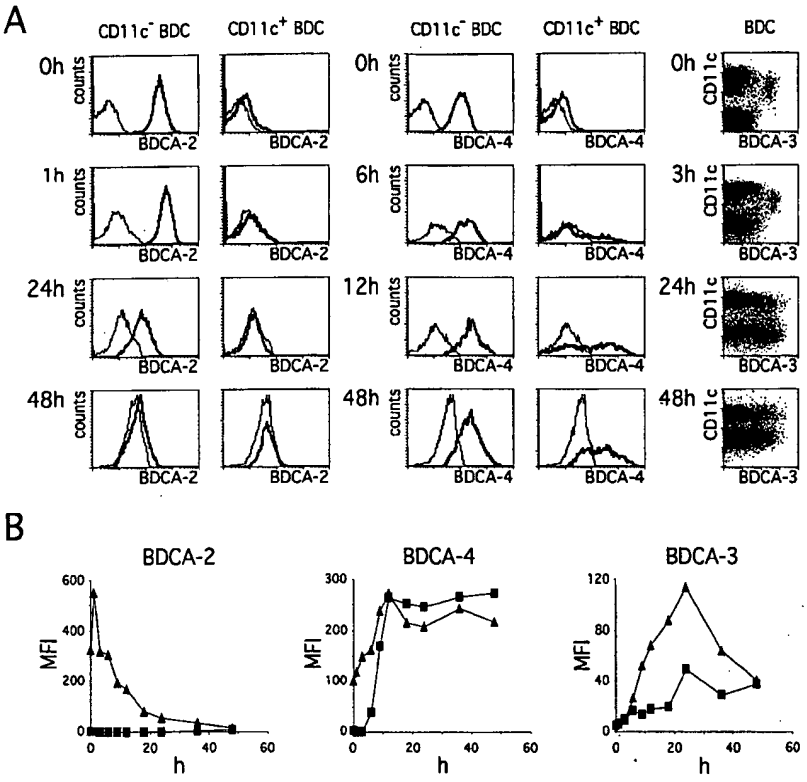

FIGURE 4. Expression of BDCA-2, BDCA-3, and BDCA-4 on purified BDC after various periods of culturing in the presence of IL-3. *A*, Histograms show staining of gated CD11c$^-$ and CD11c$^+$ BDC with PE-conjugated anti-BDCA-2 mAb (AC144) and anti-BDCA-4 mAb (AD5-17F6; bold lines) and with PE-conjugated isotype-matched control mAb (faint lines), respectively. Dot plots show staining of BDC with CD11c-PE vs anti BDCA-3 (AD5-5E8)-biotin/streptavidin-APC. *B*, Diagrams show mean fluorescence intensity (MFI) values for anti-BDCA-2-PE, anti-BDCA-4-PE, and anti-BDCA-3-biotin/streptavidin-APC staining of CD11c (▲) and CD11c$^+$ (■) BDC, respectively. For BDCA-2 and BDCA-4, MFI values were calculated by subtracting the values obtained with isotype control mAb from the values obtained with the AC144 and AD5-17F6 mAb, respectively. For BDCA-3, MFI values are calculated by subtracting the values obtained without any staining mAb (autofluorescence) from the values obtained with AD5-5E8 mAb.

Expression of BDCA-2 is completely down-regulated within 48 h on CD11c$^-$ BDC. In contrast, BDCA-4 is even further up-regulated on CD11c$^-$ BDC and, unlike BDCA-2, is also expressed to a high level on most, if not all, CD11c$^+$ BDC. Expression of BDCA-3 is rapidly induced on CD11c$^-$ BDC, reaching the highest expression level after 24 h. Thereafter, BDCA-3 expression appears to be down-regulated again. Analyzing the expression of BDCA-3 on CD11c$^+$ BDC is complicated by the fact that BDCA-3$^-$ CD11c^{bright} and BDCA-3$^+$ CD11c$^+$ subsets are present at the onset of the culture. Expression of BDCA-3 appears to remain unchanged at least until 6 h of culture on the BDCA-3$^+$ CD11c$^+$ BDC population and is induced within 3 h on at least some cells of the BDCA-3$^-$ CD11c^{bright} BDC subset.

Expression of BDCA-2, BDCA-3, and BDCA-4 on Mo-DC and CD34-derived DC

Functional CD1a$^+$ DC can be generated ex vivo from monocytes (21–23) and CD34$^+$ hemopoietic progenitor cells (24). Fig. 6 shows that immature Mo-DC (25, 26) and CD34-derived DC express CD1a, CD1c, and BDCA-4, but not BDCA-2 or BDCA-3.

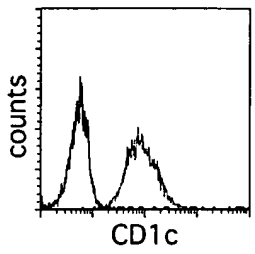

FIGURE 5. The BDCA-1-specific mAb AD5-8E7 blocks binding of the CD1c mAb M241 to MOLT-4 cells. MOLT-4 cells were preincubated with saturating amounts of AD5-8E7 mAb (bold line) or an isotope control mAb (faint line) and then stained with PE-conjugated CD1c mAb (M241).

Internalization of BDCA-2 after anti-BDCA-2 mAb labeling

The possibility that 37°C incubation of anti-BDCA-2 mAb-labeled BDCA-2$^+$ cells results in mAb internalization was addressed by staining of PBMC with FITC-conjugated AC144 mAb (IgG1). Then, following incubation at 37°C, the remaining cell surface-associated mAb was detected by staining with PE-conjugated rat anti-mouse IgG1 mAb. As shown in Fig. 7, when cells were incubated at 37°C, the intensity of the rat anti-mouse IgG1-PE staining decreased extremely rapidly to background levels. In contrast, the intensity of the AC144-FITC staining decreased only temporarily to a level of ~50%, but thereafter nearly returned to the preincubation level. This demonstrates that BDCA-2 is internalized upon anti-BDCA-2 mAb labeling, with kinetics similar to those of receptor-mediated endocytosis. The transient decrease in AC144-FITC staining intensity is probably due to patching and capping of the BDCA-2/anti-BDCA-2 mAb complex before endocytosis.

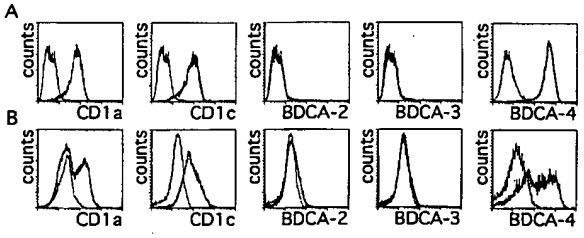

FIGURE 6. Expression of BDCA-2, BDCA-3, and BDCA-4 on immature Mo-DC and CD34-derived DC. Immature Mo-DC (*A*) and CD34-derived DC (*B*) were stained with CD1a-FITC, CD1c-PE (AD5-8E7), anti-BDCA-2-PE (AC144), anti-BDCA-3-PE (AD5-5E8), and anti-BDCA-4-PE (AD5-17F6), respectively (bold lines). The faint lines show staining with isotype control mAb. Except for the *left-most* histogram (CD1a staining), gated CD1a$^+$ cells are shown in *B*.

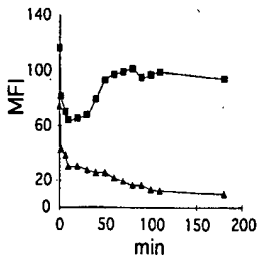

FIGURE 7. Culture of anti-BDCA-2 mAb-labeled BDCA-2$^+$ cells results in rapid mAb internalization. PBMC were labeled at 4°C with FITC-conjugated anti-BDCA-2 mAb (AC144, IgG1), incubated at 37°C for the periods indicated, and then stained at 4°C with PE-conjugated rat anti-mouse IgG1 mAb (X56) and Cy5-conjugated CD123 mAb (AC145, IgG2a). Shown are mean fluorescence intensity (MFI) values of anti BDCA-2-FITC (■) and rat anti-mouse IgG1 mAb-PE (▲) staining of gated BDCA-2$^+$ CD123$^+$ cells.

Morphology of isolated CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC

CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ cells were isolated from PBMC by MACS (Fig. 8). On May-Grünwald Giemsa staining of cytocentrifuge slides (Fig. 8), freshly isolated BDCA-2-expressing cells displayed the typical lymphoplasmacytoid morphology of CD11c$^-$ CD4$^+$ lin$^-$ DC from blood and tonsils: that is, medium-sized round cells with oval or indented nuclei (3, 6, 16). In contrast, both freshly isolated CD1c$^+$ BDC as well as freshly isolated BDCA-3$^+$ BDC displayed the typical morphological characteristics of CD11c$^+$ CD4$^+$ lin$^-$ DC from blood or tonsils: that is, less rounded cells with short cell processes and more hyperlobulated nuclei (3, 19). In addition to CD1c$^+$ BDC, CD1c$^+$ B cells with the typical morphology of small resting lymphocytes can be seen on the cytocentrifuge slides of isolated CD1c$^+$ PBMC. Highly pure CD1c$^+$ BDC were obtained if, before the enrichment of CD1c$^+$ cells, CD19$^+$ B cells were magnetically depleted from PBMC (data not shown).

Surface phenotype of CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC

The phenotypes of fresh CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC were analyzed by two-color (BDCA-2$^+$ and BDCA-3$^+$ BDC) or three-color (CD1c$^+$ BDC) immunofluorescence, respectively. The results of the phenotypic analysis are shown in Table II and can be summarized as follows. None of the BDC subsets expressed CD1a, CD8, CD15, CD16, CD19, CD20, CD23, CD25, CD27, CD34, CD61, CD69, CD71, CD77, CD80, CD83, glycophorin A, TCR$\alpha\beta$, AC133, IgD, IgM, or the CMRF-56 Ag. All BDC subsets expressed CD43, CD44, CD54, and MHC class I molecules at similar levels. BDCA-2$^+$ BDC differed from the other two subsets in that they did not express CD13, CD40, CD45RO, and CD56, but did express CD45RA and small amounts of CD10, and in that they expressed lower levels of CD18, CD38, CD58, CD98, CD116, and CLA, but higher levels of CD4. Minor proportions of BDCA-2$^+$ BDC are CD2 and CD7 positive, respectively. CD1c$^+$ BDC differ from the other two subsets in that they express higher levels of MHC class II molecules, but lower levels of CD62L, and in that they are all positive for CD2 and the Fc receptors CD32, CD64, and FcϵRI. Probably due to the Fc receptor expression, CD1c$^+$ BDC are also positive for IgG, κ and λ. Furthermore, some CD1c$^+$ BDC are positive for CD14 and CD11b, whereby the level of expression inversely correlates with the level of both CD1c and CD2 expression (data not shown). BDCA-3$^+$ BDC differ from the other two subsets in that they express CD36 at a much lower level, and they appear to express low levels of CD5. Finally, apart from

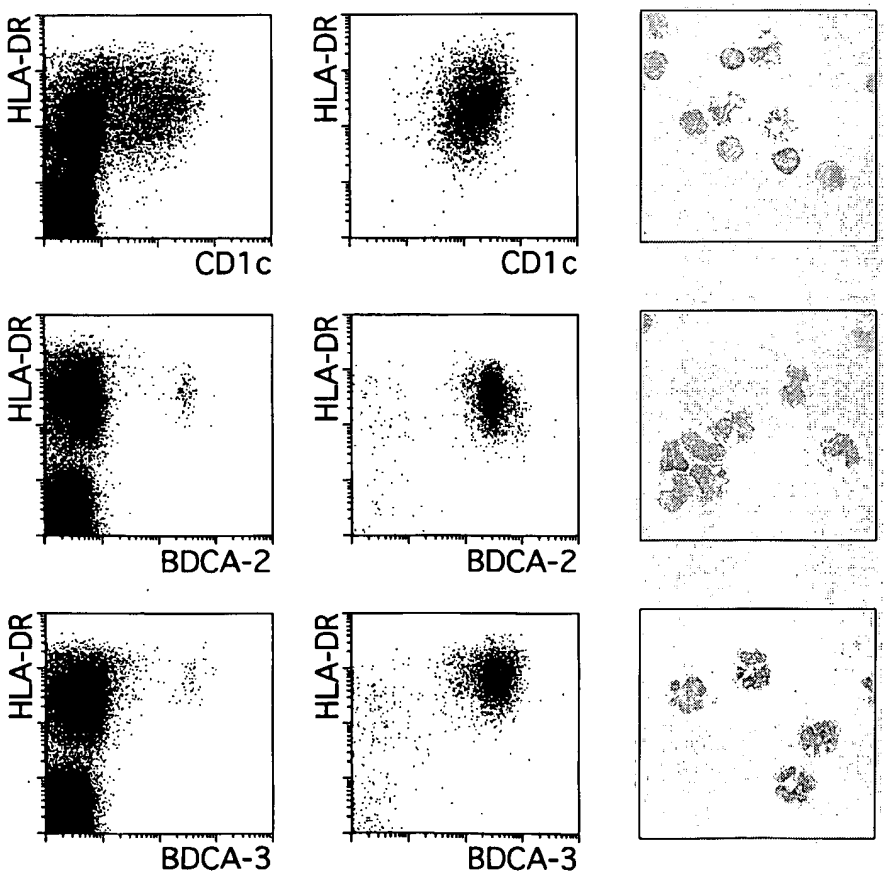

FIGURE 8. Morphology of immunomagnetically purified CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC. CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ cells were isolated from PBMC by indirect magnetic labeling with PE-conjugated primary mAb (AD5-8E7, AC144, and AD5-5E8) and anti-PE mAb-conjugated microbeads followed by enrichment of labeled cells by MACS. The dot plots show staining of PBMC with HLA-DR-FITC and the PE-conjugated mAb before (*left* dot plots) and after (*right* dot plots) magnetic enrichment of CD1c$^+$ (*upper* dot plots), BDCA-2$^+$ (*middle* dot plots), and BDCA-3$^+$ (*lower* dot plots) cells, respectively. The three pictures on the *right* show May Grünwald/Giemsa staining of isolated CD1c$^+$ (*upper* picture), BDCA-2$^+$ (*middle* picture), and BDCA-3$^+$ cells after cytocentrifugation. Note that small lymphocytes can be seen in the picture of the enriched CD1c$^+$ cells. These are CD1c$^+$ B cells.

Table II. *Surface phenotype of BDCA-2$^+$ BDC, BDCA-3$^+$ BDC, and CD1c$^+$ BDC*

Ag	Clone	BDCA-2$^+$ BDC	BDCA-3$^+$ BDC	CD1c$^+$ BDC
CD1a	HI149	–	–	–
CD1c	M241	–	–	+
CD2	S5.2	–/minor subset +	–	+
CD4	M-T321	++	+	+
CD5	CLB-T1/1, 6G4	–	–/+	–
CD7	CLB-T-3A1,7F3	–/minor subset +	–	+
CD8	SK1	–	–	–
CD10	HI10a	–/+	–	–
CD11a	G43-25B	+	ND	+
CD11b	M1/70.15.11.5	–	–	–/+
CD11c	Ki-M1	–	+	++
CD13	L138	–	+	+
CD14	TÜK4	–	–	–/+
CD15	VIMC6	–	–	–
CD16	CLB-FcR Gran/1	–	–	–
CD18	7E4	+	++	++
CD19	SJ25-C1	–	–	–
CD20	HI47	–	–	–
CD23	9P25	–	–	–
CD25	M-A251	–	–	–
CD27	M-T271	–	–	–
CD32	FLI8.26 (2003)	–	–	+
CD33	P67.6	–/+	+	++
CD34	8G12	–	–	–
CD36	AC106	+	–/+	+
CD38	HIT2	+	++	++
CD40	FC3	–	–/+	–/+
CD43	1G10	+	+	+
CD44	IM7	+	+	+
CD45RA	F8-11-13	+	–	–
CD45RO	UCHL-1	–	+	+
CD54	HA58	+	+	+
CD56	NCAM16.2	–	–/subset +	–/subset +
CD58	AICD58	+	++	++
CD61	VIPL2	–	–	–
CD62L	SK11	++	++	+
CD64	10.1	–	–	+
CD69	FN50	–	–	+
CD71	L01.1	–	–	–
CD77	38.13	–	–	–
CD80	DAL-1	–	–	–
CD83	HB15A	–	–	–
CD86	HA5.2B7	+	++	+++
CD98	HIM6	++	+++	+++
CD116	SC06	+	++	++
CD123	AC145	++	–	+
HLA-DR	AC122	+	+	++
HLA-DQ	TÜ169	+	+	++
HLA-A,B,C	W6/32	+	+	+
GPA	AC107	–	–	–
TCR$\alpha\beta$	T10B9.1A-31	–	–	–
AC133	AC133	–	–	–
FcεRI	15-1	–	–	+
IgD	TA4.1	–	–	–
IgG	Polyclonal	–	–	+
IgM	SA-DA4	–	–	–
κ	Polyclonal	–	–	+
λ	Polyclonal	–	–	+
CLA	HECA-452	++	+++	+++
CMRF44	CMRF44	–	–	–/minor subset +
CMRF56	CMRF56	–	–	–

CD11c and CD123, at least one additional Ag, CD33, is useful for discrimination of all three subsets: CD33 is expressed at low levels on BDCA-2$^+$ BDC, at intermediate levels on BDCA-3$^+$ BDC, and at high levels on CD1c$^+$ BDC.

Expression of MHC class II, CD83 and costimulatory molecules on CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC after culture

Freshly isolated CD1c$^+$ BDC and BDCA-3$^+$ BDC were cultured for 2 days in medium without any supplemented cytokines, and freshly isolated BDCA-2$^+$ BDC were cultured for 1 day in medium supplemented with IL-3 and CD40 mAb on CD32-transfected fibroblasts. After the culture period, cells were analyzed for the expression of CD1a, CD80, CD83, CD86, and HLA-DR. For the purpose of comparison, immature Mo-DC and mature Mo-DC were also included (25, 26). As shown in Fig. 9, in contrast to immature Mo-DC and mature Mo-DC, none of the BDC subsets expressed CD1a after the culture period. However, the costimulatory molecules CD80 and CD86, the dendritic cell activation Ag

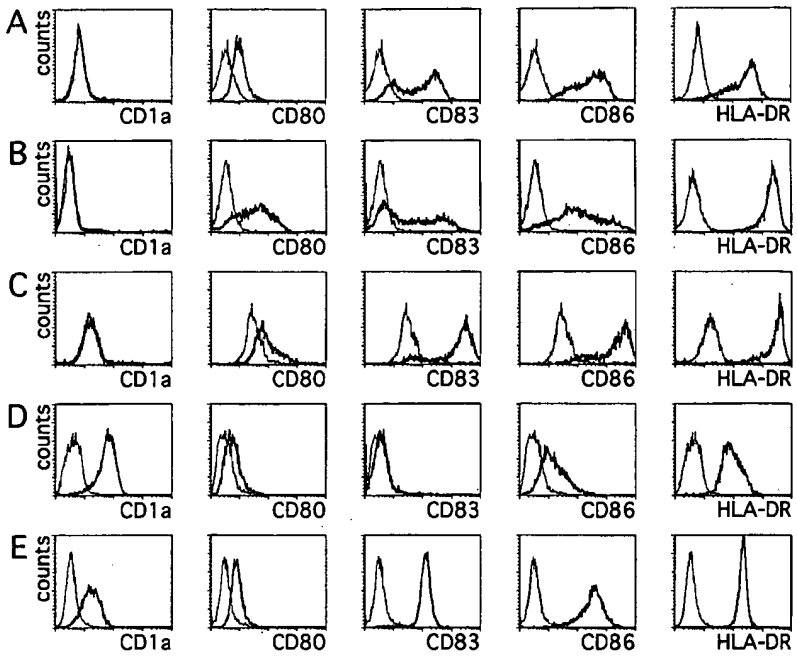

FIGURE 9. Up-regulation of MHC class II, CD83, and costimulatory molecules on CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC upon culturing. Purified CD1c$^+$ (*A*), BDCA-3$^+$ (*B*), and BDCA-2$^+$ BDC (*C*) were cultured for 2 days in medium (CD1c$^+$ and BDCA-3$^+$ BDC) or for 1 day in medium with rIL-3 and anti-CD40 mAb on CD32-transfected L cells (BDCA-2$^+$ BDC), respectively. Immature Mo-DC (*D*) and mature Mo-DC (*E*) were generated as described in *Materials and Methods*. The histograms show cell staining with CD1a-FITC, CD80-PE, CD83-PE, CD86-PE, and HLA-DR-PE, respectively (bold lines). The faint lines show cell staining with fluorochrome-matched control mAb.

CD83 (27), and HLA-DR molecules were up-regulated upon culturing all three BDC subsets to a similar level compared with mature Mo-DC. The results were not significantly different in another experiment in which all three BDC subsets were cultured for 2 days in medium supplemented with IL-3, IL-4, and GM-CSF (data not shown).

Endocytic capacity of freshly isolated CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC

The endocytic capacity of purified CD1c$^+$, BDCA-2$^+$ and BDCA-3$^+$ BDC, and, as controls, purified CD3$^+$ T cells and immature Mo-DC was examined by culturing the cells at 37°C in the presence of LY and analyzing the uptake of LY after various periods by flow cytometry. As shown in Fig. 10, unlike purified CD3$^+$ T cells, purified CD1c$^+$ BDC, BDCA-3$^+$ BDC, and to some extent BDCA-2$^+$ BDC have the ability to endocytose LY. Similar results were obtained using FITC-dextran (data not shown). The endocytic capacities of all BDC populations are much lower if compared with immature Mo-DC.

Discussion

Several attempts to generate mAb specific for human BDC have failed, yielding only mAb that bind Ags expressed by both BDC and other leukocytes. Human BDC share a large number of immunogenic cell surface structures with other blood cells. These Ags may dominate the immune response to injected BDC to a level where B cells with specificity for BDC-specific Ags are not at all or are only very rarely represented among B cells that have the capability to fuse with myeloma cells. Many investigators have tried to overcome this problem by injecting adult mice with non-BDC and cyclophosphamide to ablate B cells with specificity for shared Ags or by injecting neonatal mice with non-BDC to tolerize B cells with specificity for shared Ags (28, 29). Here, we have approached this problem with a recently described contralateral footpad immunization procedure (30). This system is based on the fact that naive Ag-specific T and B cells continuously recirculate among peripheral lymphoid organs as long as they do not encounter Ag (31–34), but become immediately retained within a peripheral lymphoid organ for several days, if not weeks, once they are activated by Ag (35–38). Therefore, in our example, the left footpads of mice were injected on days −3, 0, 4, 7, 11, and 14 with Bristol-8 B lymphoblastoma cells, while the right footpads were injected with BDC on days 0, 4, 7, 11, and 14. Naive B and T cells with specificity for shared Ags, e.g., HLA class II molecules, should become activated by Bristol-8 cells between days −3 and 0 in the left popliteal lymph node and thereupon be retained there, while all lymphocytes with specificity for Ags unique to BDC should remain available for activation after day 0 in the right popliteal lymph node.

This immunization technique combined with a powerful procedure for rapid isolation of large numbers of BDC has permitted us to produce a panel of mAb that recognize three presumably novel BDC Ags: BDCA-2, BDCA-3, and BDCA-4. We found that in noncultured human blood BDCA-2 and BDCA-4 are exclusively expressed by a CD123^{bright} CD11c$^-$ DC population. This DC population is now commonly referred to as plasmacytoid BDC (4–7, 10, 14, 16). Using BDCA-2 or BDCA-4 as a surface marker for immunomagnetic isolation and/or flow cytometric identification of plasmacytoid BDC, our results on frequency, immunophenotype,

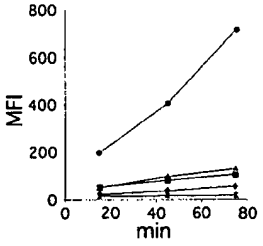

FIGURE 10. Endocytic capacity of freshly isolated CD1c$^+$, BDCA-2$^+$, and BDCA-3$^+$ BDC in comparison with purified CD3$^+$ T cells and immature Mo-DC. CD1c$^+$ BDC (▲), BDCA-2$^+$ BDC (◆), BDCA-3$^+$ BDC (■), CD3$^+$ T cells (*), and immature Mo-DC (●) were incubated at 37°C in medium with 1 mg/ml LY for the periods indicated, washed three times in ice-cold PBS/EDTA/BSA, and then analyzed by flow cytometry. Shown are the mean fluorescence intensity (MFI) values for LY fluorescence after subtracting the MFI values that were obtained upon incubation at 4°C in the absence of LY.

morphology, endocytic capacity, and maturation of these cells were consistent with most previous reports where a large panel of leukocyte Ags was used (3–8, 10, 14). This clearly illustrates that both Ags are useful markers for plasmacytoid BDC in noncultured human blood. Preliminary results from stainings of tonsilar cells indicate that the T cell zone-associated plasmacytoid DC in peripheral lymphoid organs can also be discriminated from other lymphoid tissue-associated DC populations, such as germinal center DC, interdigitating DC, and follicular DC based on the expression of BDCA-2 and BDCA-4 (data not shown).

Concerning this point it is interesting that, unlike BDCA-2, BDCA-4 is also expressed on several in vitro differentiated DC populations: 1) in contrast to BDCA-2, BDCA-4 is expressed on both Mo-DC and CD34-derived DC; 2) whereas expression of BDCA-2 is completely down-regulated on plasmacytoid BDC once they have undergone IL-3-mediated maturation in culture, expression of BDCA-4 is, in fact, up-regulated on cultured plasmacytoid BDC; and 3) in contrast to BDCA-2, BDCA-4 is expressed within 12 h by a majority of cultured $CD11c^+$ BDC, although it is unclear whether this is only true for the larger $CD1c^+$ $CD11c^{bright}$ population or is also true for the smaller $CD1c^-$ $CD11c^+$ $CD123^-$ population. The finding that no other BDCA-4^+ cells than plasmacytoid BDC appear to be present in noncultured human blood, in fact, indicates that no counterparts of the in vitro differentiated BDCA-4^+ DC populations are present in blood.

Labeling of BDCA-2 by anti-BDCA-2 mAb and incubation at 37°C induce rapid internalization of the Ag-mAb complex. In analogy to other endocytic receptors on DC that are down-regulated upon maturation, such as langerin (39), it is tempting to speculate that BDCA-2 may be a receptor with Ag capture function.

Expression of BDCA-3 was found to be restricted to a small population of $CD1c^-$ $CD11c^+$ $CD123^-$ BDC in noncultured human blood. With respect to phenotype, morphology, endocytic capacity, and maturation requirements, this BDC population is quite similar to the $CD1c^+$ $CD11c^{bright}$ $CD123^{dim}$ BDC population. However, apart from BDCA-3 and CD1c expression, our immunophenotypic analysis has revealed some striking differences: in contrast to $CD1c^+$ BDC, BDCA-3^+ BDC do not express the Fc receptors CD32, CD64, and FcεRI, and they do not express CD2. The lack of Fc receptor expression indicates that BDCA-3^+ BDC, unlike $CD1c^+$ BDC (40, 41), do not have the capability of Ig-mediated Ag uptake.

In principle, BDCA-3^+ BDC and $CD1c^+$ BDC may represent maturational stages of the same cell type or unrelated DC types. The fact that BDCA-3 expression is induced on a reasonable proportion of $CD1c^+$ BDC after culture-induced maturation may be considered an argument in favor of the former concept, but because the same observation was also made for IL-3-stimulated plasmacytoid BDC, such data could also be taken as an argument in favor of a similar relationship between BDCA-3^+ BDC and plasmacytoid BDC.

A recent study by Ito et al. (7) has provided evidence that $CD1c^+$ $CD11c^{bright}$ BDC, in contrast to $CD1c^-$ $CD11c^+$ BDC, have the capacity to acquire Langerhans cell characteristics (expression of Lag Ag, E-cadherin, and langerin, and presence of Birbeck granules) when cultured with GM-CSF, IL-4, and TGF-β1. If BDCA-3^+ BDC and $CD1c^+$ BDC represent maturational stages of the same cell type, this would indicate that BDCA-3^+ BDC have either already lost or not yet acquired the capacity to differentiate into Langerhans cells.

In contradiction to our results, Ito et al. (7) have reported that $CD1c^+$ $CD11c^{bright}$ BDC, unlike $CD1c^-$ $CD11c^+$ BDC, express CD1a. This is, in fact, more than doubtful. The authors stated that they have used the two mAb, BL-6 and B-B5, for staining of CD1a and that a difference in staining intensity was actually observed when the two mAb were compared (staining with B-B5 was probably brighter). We found that staining of BDC was clearly negative using optimal titers of the CD1a mAb BL-6 and HI149, but positive using B-B5. Moreover, it turned out that B-B5, unlike BL-6 and HI149, stained a high proportion of $CD19^+$ B cells in blood. Thus, the staining pattern of B-B5 was quite reminiscent of a CD1c mAb rather than a CD1a mAb, and, in fact, we could show that our CD1c mAb AD5-8E7 inhibits binding of B-B5 to MOLT-4 cells (data not shown). Therefore, we conclude that B-B5 recognizes CD1c and that $CD1c^+$ BDC do not express CD1a.

Staining of $CD1c^+$ BDC for CD1c, CD2, and CD14 revealed that a minor proportion of BDC expresses CD14 to a variable degree and that the level of CD1c as well as CD2 expression on these cells is inversely proportional to the level of CD14 expression (data not shown). This observation is in accordance with a linear differentiation model, in which $CD1c^+$ $CD2^+$ $CD11c^{bright}$ $CD14^-$ BDC are the progeny of $CD14^+$ $CD1c^-$ $CD2^-$ monocytes rather than the progeny of a common precursor of both cell types. This concept finds further support in the observation of Crawford et al. (42) that a considerable proportion of $CD14^+$ monocytes already expresses very low levels of CD2 and has the capacity to rapidly differentiate into mature DC with typical dendritic morphology and potent T cell stimulatory function when cultured with GM-CSF and IL-4.

In summary, the results of this study identify three presumably novel markers of BDC in human blood: 1) BDCA-2, 2) BDCA-4 for $CD11c^-$ $CD123^{bright}$ plasmacytoid BDC, and 3) BDCA-3 for $CD11c^+$ $CD123^-$ BDC. A third population of $CD11c^{bright}$ $CD123^{dim}$ BDC can be identified based on the expression of CD1c and the lack of B cell lineage Ags. It is noteworthy that the expression profile of BDCA-2, BDCA-3, and BDCA-4 is virtually indistinguishable on all three subsets after maturation in culture. The use of CD1c (BDCA-1), BDCA-2, BDCA-3, and BDCA-4 mAb provides a convenient and efficient way to rapidly detect, enumerate, and isolate BDC populations from PBMC, leukapheresis material, or whole blood without apparent functional perturbation. This will be a valuable aid for their further functional and molecular characterization and may prove useful in elucidating their interrelationships. Furthermore, the ability to easily isolate BDC populations to homogeneity will greatly facilitate their clinical use.

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Exhibit D

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Regular Article

Identification and Characterization of the Gene for a Novel C-Type Lectin (*CLECSF7*) That Maps near the Natural Killer Gene Complex on Human Chromosome 12[*1]

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Abstract

We report the identification and characterization of a novel C-type lectin gene, named *HECL* (HGMW-approved symbol *CLECSF7*), that maps close to the natural killer gene complex on human chromosome 12p13. Sequence analysis revealed a complete open reading frame of 549 bp comprising several putative glycosylation and phosphorylation sites as well as a C-terminal C-type carbohydrate-recognition domain. Homology analysis revealed that HECL exhibits a significant degree of divergence from the natural killer cell receptors that comprise the natural killer gene complex. These natural killer cell receptors all belong to group V of the C-type lectin superfamily. *HECL,* however, is most closely related to the sole group II C-type lectins reported to map near this region of the genome, the murine *Nkcl* and *Mpcl* genes. Like *Nkcl, HECL* is expressed in a variety of hematopoietic cell types and has a complete Ca^{2+}-binding site 2. Despite the presence of critical amino acids for sugar binding in Ca^{2+}-binding site 2, HECL does not seem to bind carbohydrate. Moreover, *HECL* is the first non-receptor-like C-type lectin to map near the natural killer gene complex.

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BDCA-2, a Novel Plasmacytoid Dendritic Cell–specific Type II C-type Lectin, Mediates Antigen Capture and Is a Potent Inhibitor of Interferon α/β Induction

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Abstract

Plasmacytoid dendritic cells are present in lymphoid and nonlymphoid tissue and contribute substantially to both innate and adaptive immunity. Recently, we have described several monoclonal antibodies that recognize a plasmacytoid dendritic cell-specific antigen, which we have termed BDCA-2. Molecular cloning of BDCA-2 revealed that BDCA-2 is a novel type II C-type lectin, which shows 50.7% sequence identity at the amino acid level to its putative murine ortholog, the murine dendritic cell–associated C-type lectin 2. Anti–BDCA-2 monoclonal antibodies are rapidly internalized and efficiently presented to T cells, indicating that BDCA-2 could play a role in ligand internalization and presentation. Furthermore, ligation of BDCA-2 potently suppresses induction of interferon α/β production in plasmacytoid dendritic cells, presumably by a mechanism dependent on calcium mobilization and protein-tyrosine phosphorylation by src-family protein-tyrosine kinases. Inasmuch as production of interferon α/β by plasmacytoid dendritic cells is considered to be a major pathophysiological factor in systemic lupus erythematosus, triggering of BDCA-2 should be evaluated as therapeutic strategy for blocking production of interferon α/β in systemic lupus erythematosus patients.

Key words: interferon type I • monoclonal antibodies • magnetic cell sorting • interferon inducers • systemic lupus erythematosus

Introduction

Plasmacytoid dendritic cells (PDCs),* which are also known as plasmacytoid T cells (1), plasmacytoid monocytes (2), natural IFN-α/β–producing cells (natural IPCs; references 3–10), and type 2 predendritic cells (pDC2; references 11–14), constitute a subset of immature dendritic cells (DCs), which is capable of differentiating in vitro into mature DCs with typical dendritic cell morphology and potent T cell stimulatory function when exposed to IL-3 alone (15–17), IL-3 and CD40L (18, 19), viruses (20, 21), and bacterial DNA containing unmethylated CpG motifs (CpG-DNA; references 14 and 22). Immature PDCs have been found in human LN (23), tonsils (18, 24), peripheral blood (16, 17, 19, 24–27), cord blood (13, 28), adult and fetal thymus (13, 29–31), fetal liver (13), and fetal bone marrow (13, 24). In addition, presence of PDCs in affected and sometimes even nonaffected tissue has been described in association with many pathological circumstances, including granulomatous lymphadenitis (32), Kikuchi's lymph-

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*Abbreviations used in this paper: CRD, carbohydrate recognition domain; DC, dendritic cell; DCIR, human DC immunoreceptor; dectin-2, DC-associated C-type lectin 2; ds, double-stranded; FLU, influenza A/PR8 virus; PDC, plasmacytoid dendritic cell; RT, reverse transcription; SLE, systemic lupus erythematosus; ss, single-stranded; TBE, Tris-borate-EDTA; TLR9, toll-like receptor 9.

adenitis (33), dermal lesions and noninflammatory skin of systemic lupus erythematosus (SLE) patients (34, 35), benign cutaneous lymphoid infiltrates (36, 37), nasal mucosa of allergic as well as nonallergic individuals (38), and cerebrospinal fluid from patients with inflammatory as well as noninflammatory neurological diseases (39). Identification of immature PDCs in blood has been commonly based on a multitude of immunophenotypic criteria, such as the absence of CD11c, ILT-1, and a panel of leukocyte lineage-specific Ags (e.g., CD3, CD14, CD19, and CD56) and the presence of CD4, CD123, HLA-DR, CD68, ILT3, or CD45RA (10, 16, 17, 24–27). However, using a panel of new mAb raised against blood DCs, we have recently identified two novel markers of PDCs (19), BDCA-2 and BDCA-4, which enable direct identification of PDCs in human blood. Here we describe the molecular and functional characterization of BDCA-2. BDCA-2 is a novel type II C-type lectin presumably involved in ligand internalization, processing and presentation, as well as in inhibition of IFN-α/β synthesis in PDCs. Our results identify BDCA-2 as an attractive pharmaceutical target for specific downmodulation of IFN-α/β expression in patients with SLE.

Materials and Methods

Cell Preparations. Buffy coats from normal healthy volunteers were obtained from the Institute for Transfusion Medicine, Merheim Hospital, Cologne, Germany. PBMCs were prepared from buffy coats by standard Ficoll-Paque (Amersham Pharmacia Biotech) density gradient centrifugation. PDCs were isolated from PBMCs by direct magnetic labeling with anti–BDCA-4 mAb (AD5–17F6)–conjugated microbeads (BDCA-4 Cell Isolation Kit; Miltenyi Biotec; reference 19) and enrichment of labeled cells using a high-gradient magnetic cell sorting device (MiniMACS®; Miltenyi Biotec). As determined by staining with PE-conjugated anti–BDCA-2 mAb (AC144; Miltenyi Biotec; reference 19) and flow cytometric analysis using a FACSCalibur™ (Becton Dickinson) and CELLQuest™ software (Becton Dickinson), PDC purities of 92–98% were routinely obtained.

For molecular cloning of BDCA-2, PDCs were isolated from leukapheresis products by indirect magnetic labeling with FITC-conjugated anti–BDCA-2 mAb (AC144; Miltenyi Biotec) and anti-FITC mAb-conjugated microbeads (Miltenyi Biotec), followed by enrichment of labeled cells using a clinically approved immunomagnetic selection device (CliniMACS®; Miltenyi Biotec). Leukapheresis products from nonmobilized volunteers were obtained from the DRK-Blutspendezentrale Ulm, Ulm, Germany.

For reverse transcription (RT)-PCR analysis of BDCA-2 mRNA expression, T cells, B cells, NK cells, and monocytes were isolated from PBMCs by cell sorting with a FACSVantage™ (Becton Dickinson) using mAb against CD3 (mAb SK9, IgG2b; Becton Dickinson), CD19 (mAb 4G7, IgG1; Becton Dickinson), CD56 (mAb MY31, IgG1; Becton Dickinson), and CD14 (mAb MϕP9, IgG2b; Becton Dickinson), respectively. Similarly, $CD11c^+$ myeloid DCs and $CD123^+$ PDCs were isolated by FACS® based on the absence of a panel of leukocyte lineage-specific Ag (Lineage Cocktail 1 from Becton Dickinson including mAb against CD3, CD14, CD16, CD19, CD20, and CD56), and the presence of HLA-DR (mAb L243, IgG2a; Becton Dickinson) and either of CD11c (mAb S-HCL-3, IgG2b; Becton Dickinson) or of CD123 (mAb 9F5, IgG1; Becton Dickinson), respectively.

Expression Cloning of Full-length BDCA-2 cDNA. $Poly(A)^+$ RNA was isolated from magnetically purified PDC using Oligo(dT) microbeads and a high-gradient magnetic cell sorting device (μMACS® mRNA Isolation Kit; Miltenyi Biotec), and used to construct a cDNA library in pEF18SR, a modified pEF18S expression vector (40). Transient expression was performed in COS1 cells. After several rounds of MACS® and finally FACS® using anti BDCA-2 mAb AC144 (19), plasmid DNA of a positive clone (H4#10A) was purified and sequenced. Homology searches were performed in GenBank/EMBL/DDBJ nucleotide databases. Alignments with the murine DC-associated C-type lectin 2 (dectin-2; reference 41) and the human DC immunoreceptor (DCIR; reference 42) and several other type II C-type lectins were analyzed using the ALIGN function of the Genestream server at the Institut de Génétique Humaine, Montpellier, France.

RT-PCR Analysis. For RT-PCR analysis of BDCA-2 mRNA expression in various blood cell populations, $poly(A)^+$ RNA was isolated from FACS®-purified human T cells, B cells, NK cells, monocytes, $CD11c^+$ myeloid blood DCs, and $CD11c^-$ PDCs. First-strand cDNA was prepared from isolated $poly(A)^+$ RNA by reverse transcription using Oligo(dT)12–18 primers (Life Technologies) and the SuperScript II reverse transcriptase (Life Technologies). For RT-PCR analysis of BDCA-2 mRNA expression in various tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, LN, bone marrow, and fetal liver) sets of normalized first-strand cDNA (Human Multiple Tissue cDNA Panels I and II; CLONTECH Laboratories, Inc.) were used. All cDNAs were normalized to several different housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, phospholipase A2, α-tubulin, and β-actin). PCR amplification (34 or 38 cycles) was performed using AdvanTaq Plus DNA Polymerase (CLONTECH Laboratories, Inc.) and the primers 5′-TTG AAA GAA CCA CAC CCC GAA AGT-3′ (forward primer) and 5′-TAG CTT TCT ACA ACG GTG GAT GCC-3′ (reverse primer) under the following conditions: 30 s denaturation at 94°C and 120 s annealing and elongation at 68°C. The PCR products were size-fractionated by agarose gel electrophoresis and analyzed after ethidium bromide staining.

Cloning of Truncated BDCA-2 cDNAs (Splice Variants). Purified $Poly(A)^+$ RNA from PDCs was subjected to DNase I treatment and BDCA-2 mRNA species were specifically amplified by RT-PCR using the Titan One Tube RT-PCR-System (Roche Diagnostics GmbH) and the primers 5′-ACA CAA TGG TGC CTG AAG AAG-3′ (forward primer) and 5′-ACA TTT TAG GGG CAT TCC TTG TA-3′ (reverse primer). After preincubation for 10 min at 25°C and for 30 min at 55°C, PCR cycles (20, 25, and 30 cycles) were run under the following conditions: 30 s denaturation at 94°C, 45 s annealing at 57°C, and 90 s elongation at 72°C. Amplified cDNA fragments were size-fractionated by 4–12% Tris-borate-EDTA (TBE) PAGE and analyzed after staining with ethidium bromide. Individual bands were excised and subjected to PCR reamplification (30 cycles) using the same primer set and the same cycle conditions. After another round of size-fractionation by 4–12% TBE PAGE and excision of the appropriate bands, reamplified fragments were cloned and sequenced. To confirm that two different truncated BDCA-2 mRNA species lacking either exon 3 (665 bp) or exon 4 (647 bp) were present in one excised band (Fig. 4), another round of PCR reamplification (30 cycles; 30 s denaturation at 94°C, 60 s annealing at 60°C, and

60 s elongation at 72°C) was performed using two new sets of primers that allow for amplification of only the variant lacking exon 3 (forward primer binding in exon 2: 5′-ACA CAA TGG TGC CTG AAG AAG-3′ and reverse primer binding in exon 4: 5′-TGG ACA GCC TCT TGA CAG TTT-3′) or the variant lacking exon 4 (forward primer binding in exon 3: 5′-TGG CAG TCG TAT CCA TCT TG-3′; reverse primer binding in exon 7: 5′-ACA TTT TAG GGG CAT TCC TTG TA-3′).

Immunohistological Localization/Characterization of BDCA-2–expressing PDCs. Immunohistochemical staining of air-dried, acetone-fixed sections from LNs, tonsils, thymus, and testis with anti–BDCA-2 mAb (AC144, IgG1) was done following an indirect immunoperoxidase technique. Single-color immunofluorescent staining of acetone-fixed cryosections of lymph nodes and thymus was done by primary staining with nonconjugated anti–BDCA-2 mAb (AC144, IgG1) or CD123 mAb (7G3, IgG2a; Becton Dickinson) and secondary staining with FITC-conjugated anti–mouse IgG1 Ab (Southern Biotechnology Associates, Inc.) and Texas red–conjugated anti–mouse IgG2a Ab (Southern Biotechnology Associates, Inc.), respectively.

Double–color immunofluorescent staining of cryostat sections of frozen human tonsils was performed using anti–BDCA-2-FITC (AC144, IgG1) and nonconjugated mAb (IgG2) against CD8, CD20, CD123, and HLA-DR, respectively. Staining with nonconjugated primary mAb was revealed by secondary staining with biotinylated anti-isotype mAb and Texas red–conjugated streptavidin.

Immunoprecipitation. Cells were surface labeled with 1 mCi of $Na^{125}I$ using the sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate method (43). After surface labeling, cells were lysed in 1% Triton X-100, 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After overnight preclearing with protein G-sepharose, lysates were incubated with anti–BDCA-2 mAb (AC144, IgG1) or isotype-matched control mAb at +4°C for 4 h, and immune complexes were precipitated by addition of protein G-Sepharose for 1.5 h at +4°C. Precipitates were washed three times with lysis buffer, followed by a final wash with 10 mM Tris-HCl, pH 7.4, 15 mM NaCl, and then resuspended in nonreducing or reducing sample buffer. SDS-PAGE analysis was performed according to a standard procedure. After the run, gels were dried and exposed to autoradiography film (Amersham Pharmacia Biotech) for 2–5 d.

Measurement of Cytosolic Calcium Concentrations ($[Ca^{2+}]i$). PDCs, U937 cells, and BDCA-2–transfected U937 cells were loaded with Indo-1 AM (Sigma-Aldrich) as described (44). In stimulation experiments, anti–BDCA-2 mAb (AC144, IgG1) was added to PDCs, U937 cells, and BDCA-2–transfected U937 cells, respectively, followed by $F(ab')_2$ goat anti–mouse IgG (Milan Analytica) as cross-linker. Cells were then analyzed on a flow cytofluorimeter (FACSVantage™; Becton Dickinson) to detect Ca^{2+} fluxes. Only live (based on forward scatter criteria) and Indo-1–loaded cells (based on 405 nM versus 525 nM emission spectra) were included in the analysis. To test whether a $[Ca^{2+}]i$ increase was dependent on extracellular Ca^{2+}, cells were stimulated in buffer with excess EGTA (4 mM). To test whether a $[Ca^{2+}]i$ increase was dependent on src-family protein-tyrosine kinases, cells were preincubated for 40 min with 20 μg/ml PP2 (Calbiochem).

Western Blotting. For anti-phosphotyrosine blots, PDCs, BDCA-2–transfected U937 cells, and BDCA-2–transfected Jurkat cells (10^6) were incubated for 2 min at 37°C with medium or with anti–BDCA-2 mAb (AC144, IgG1). Cells were washed in ice-cold PBS and lysed in Laemmli protein sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerine, 0.005% bromphenol blue). Cell lysates were sonicated, boiled, separated by SDS-PAGE, transferred to nitrocellulose, and probed with horseradish peroxidase (HRP)-coupled anti-phosphotyrosine mAb PY20 (100 ng/ml; Becton Dickinson). Immunoblotted proteins were visualized by chemiluminescence using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech).

Ag Presentation Assay. 30,000 cells of the B13 T cell clone (45) were cocultured with (a) 37,000, (b) 18,500, (c) 9,250, (d) 4,625, (e) 2,312, and (f) 1,156 irradiated PDCs (3,000 rad) in 96-well flat-bottom microplates in the presence of 200 ng/ml of IgG1 mAb. mAb used in the assay were the following: anti–BDCA-2 (AC144, IgG1), anti–ILT3 (ZM3.8, IgG1), and anti-cytokeratin (CK3–11D5, IgG1). After 48 h, the cultures were pulsed with [^{3}H]thymidine (1 μCi/well, specific activity 5 Ci/mmol), and the radioactivity incorporated was measured after additional 16 h.

In Vitro Stimulation of PDCs. Purified PDCs were cultured at a cell density of 5×10^5 to 10^6 cells/ml in medium (RPMI 1640 supplemented with 2 mM L-glutamine, 10% FCS, 110 mg/liter sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C in a humidified 5% CO_2-containing atmosphere in the presence of 10 ng/ml IL-3 (PeproTech) for 24 h. One of the following agents was typically added for induction of IFN-α/β expression: (a) influenza A/PR8 virus (FLU, used at a final concentration of 5 hemagglutinating units/ml), (b) anti–single-stranded (ss) and double-stranded (ds) DNA mAb MER-3 (Serotec; dialyzed against RPMI 1640 and used at a final concentration of 2.5 μg/ml) plus plasmid pcDNA3 (used at a final concentration 0.5 μg/ml), (c) sera from SLE patients (provided by Falk Hiepe, Charite, Medical School of the Humboldt University, Berlin, Germany, and used at a final dilution of 1/5), (d) CpG oligonucleotides (AAC-30 and GAC-30; reference 14), and (e) OK432, an immunomodulator derived from the Su strain of *Streptococcus pyogenes*. To test the effect of triggering of BDCA-2 on the induction of IFN-α/β expression, anti–BDCA-2 mAb (AC144, IgG1) was added at a final concentration of 5 μg/ml.

ELISA. The levels of IFN-α in culture supernatants were determined by an ELISA kit (R&D Systems).

Results

BDCA-2 Is a Type II C-type Lectin. We used transient expression cloning in COS1 cells to identify BDCA-2, the molecule recognized by the PDC-specific mAb AC144 (19). A full-length 1,313 bp cDNA sequence was isolated after transfection of COS1 cells with a cDNA library prepared from purified PDCs. The cDNA contains a 491 bp 5′ UTR, a 642 bp open reading frame, a 3′ UTR of 150 nucleotides, and a poly(A) tail of 30 bp (GenBank/EMBL/DDBJ accession no. AF293615). The open reading frame predicts a novel polypeptide of 213 amino acids (Fig. 1) with a molecular mass of 25.021 kD. The presence of a hydrophobic signal anchor (residues 22–41) indicates a type II transmembrane protein. BDCA-2 belongs to the Ca^{2+}-dependent (C-type) lectin family with a single carbohydrate recognition domain (CRD; Fig. 1) featuring a glutamate-proline-asparagine (EPN) motif (position 172–174) predicting mannose-type specificity (46). Three potential N-glycosylation sites are present at positions 110–113, 137–140, and 164–167 amino acids, respectively. Finally, BDCA-2 contains an intracellular

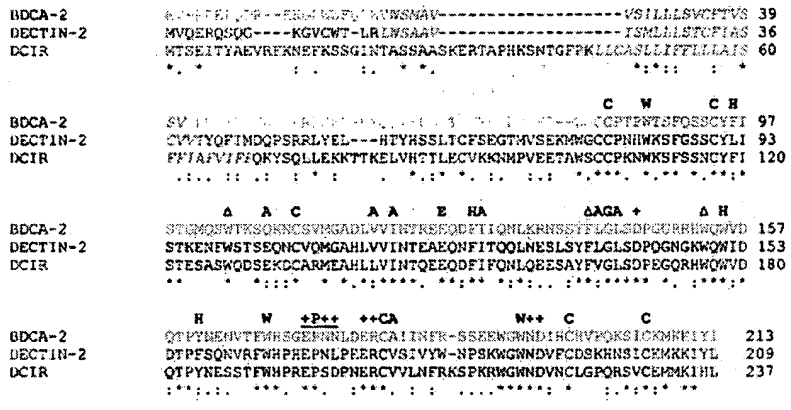

Figure 1. Amino acid sequence alignment of the type II C-type lectins BDCA-2, murine dectin-2, and human DCIR. Identical or conserved residues are indicated by (*), conserved and semiconserved substitutions by (:) and (.), respectively; the putative transmembrane domains are shown in red italics; the shaded area denotes the CRD and the residues strongly conserved among C-type lectins are shown in bold type (H, hydrophobic; A, aliphatic; C, cysteine; G, glycine; E, glutamic acid; W, tryptophan; Δ, aromatic; +, involved in calcium-dependent binding of carbohydrate; +P++, so-called EPN-motif predicting mannose-type specificity [reference 46]).

domain of 21 amino acids without any known motif implicated in signal transduction.

Amino acid sequence comparison in the GenBank and EMBL databases revealed marked homology between BDCA-2 and several type II C-type lectins, especially in the CRD. BDCA-2 displays highest homology with murine dectin-2 (50.7% amino acid identity in the overall sequence; reference 41) and human DCIR (42.1% amino acid identity in the overall sequence; reference 42; Fig. 1).

Genomic Structure and Chromosomal Localization of the Human BDCA-2 Gene. Due to complete nucleotide sequence identity over seven stretches between the BDCA-2–encoding cDNA and sequences of genomic DNA fragments present in the Genbank database (GenBank/EMBL/DDBJ accession no. AC006517: clone RP11–277J24, working draft sequence, 21 unordered pieces), the human BDCA-2 gene could be mapped to the short arm of chromosome 12. Furthermore, by analyzing the ends of the seven sequence identity stretches (the putative exon-intron boundaries) for the presence of conserved splice donor (5′(C/A)AG-GU(A/G)AGU) and acceptor sites (5′(U/C)11N(U/C)AG-(G/A)) (47), and by sequencing a series of truncated BDCA-2 mRNA species (see below), the number of exons in the full-length BDCA-2 mRNA as well as the precise positions of intron excisions, could be deduced. The full-length BDCA-2 mRNA consists of seven exons, with exon 1 (1–361 bp) representing most of the 5′ UTR, exon 2 (362–522 bp) encoding 10 amino acids of the cytoplasmic domain, exon 3 (523–615 bp) encoding 31 amino acids mainly in the transmembrane domain, exon 4 (616–726 bp) encoding 37 amino acids mainly in the neck domain, exon 5 (727–872 bp) encoding 49 amino acids mainly in the CRD, exon 6 (873–988 bp) encoding 39 bp in the CRD, and exon 7 (989–1283 bp) encoding 47 bp in the CRD. The overall exon-intron organization of BDCA-2 and dectin-2 (41) are virtually identical, suggesting that dectin-2 may indeed represent the murine homologue of human BDCA-2 rather than the homologue of any other human C-type lectin.

BDCA-2 Is Selectively Expressed in PDCs. Using first-strand cDNA from multiple tissues and blood leukocyte populations for analyzing BDCA-2 mRNA expression by PCR (34 cycles, forward primer in the 5′UTR and a reverse primer in the 3′UTR), BDCA-2 transcripts were easily detectable in purified PDCs, but not detectable in all other tested blood leukocyte populations, including $CD11c^+$ myeloid DCs, and in all tested tissues (Fig. 2). This finding provides evidence that other cells than PDCs most likely do not express BDCA-2 mRNA. On the other hand, this finding also raises the question of whether PDCs in tissues do not express BDCA-2 or whether the PCR analysis has not been sensitive enough to detect PDC-derived BDCA-2 mRNA in first-strand cDNA from whole tissues. To further analyze this, four more PCR cycles (38 instead of 34) were used for PCR amplification of BDCA-2 mRNA. Indeed, using four more PCR cycles, weak BDCA-2 mRNA signals were also detectable in some but not all tissues (tonsil, bone marrow, pancreas, testis, and ovary; data not shown). To analyze to which degree BDCA-2 mRNA expression in these tissues might be attributable to presence of BDCA-2–expressing PDCs, sections from several tissues were stained with anti–BDCA-2 mAb. Double-staining of sections from tonsils for BDCA-2 (green) and CD8, CD20, CD123, or HLA-DR (red), respectively, revealed that BDCA-2 expression is completely restricted to $CD123^+HLA\text{-}DR^+CD8^-CD20^-$ PDCs. In fact, confirming previous observations about the localization of PDCs in tonsils (18), $BDCA\text{-}2^+CD123^+HLA\text{-}DR^+CD8^-CD20^-$ PDCs were found in the T cell–rich extrafollicular areas but not within germinal centers (Fig. 3). Anti–BDCA-2 mAb also strongly labeled large clusters

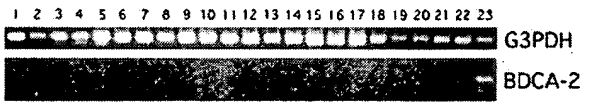

Figure 2. Expression analysis of BDCA-2 mRNA by RT-PCR (34 PCR-cycles) on various tissues and cell populations (1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, testis; 12, ovary; 13, small intestine; 14, LN; 15, bone marrow; 16, fetal liver; 17, tonsil; 18, T cells; 19, B cells; 20, NK cells; 21, monocytes; 22, $CD11c^{bright}CD123^{low}$ myeloid DCs (reference 19); 23, $CD11c^-CD123^{bright}$ PDCs). All cDNAs were normalized using four housekeeping genes including glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

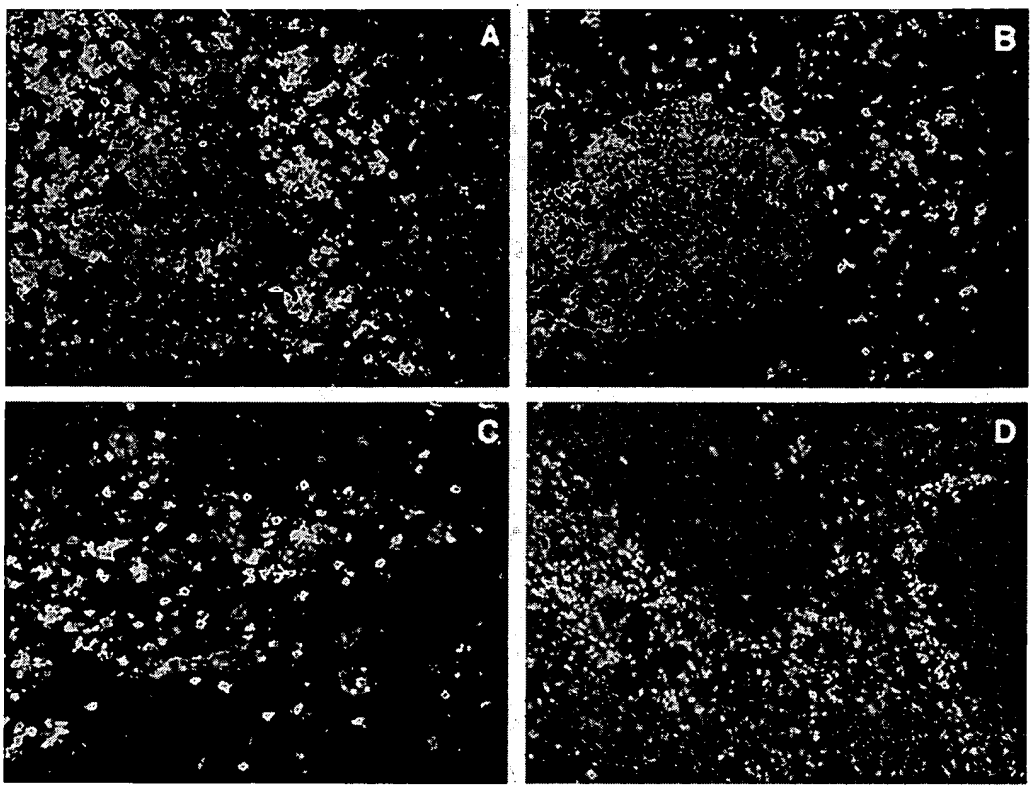

Figure 3. Anatomical localization of BDCA-2–expressing PDCs in inflamed tonsils. Fluorescent double staining with FITC–conjugated (green) anti–BDCA-2 mAb and Texas Red–conjugated (red) CD8 mAb (A), CD20 mAb (B), CD123 mAb (C), and anti–HLA-DR mAb (D). Note that BDCA-2–expressing PDCs are found in the T cell-rich extrafollicular areas but not within the germinal center. Like CD123, BDCA-2 is expressed on PDCs, but unlike CD123, BDCA-2 is not expressed on HEVs. One representative experiment of three is shown.

of $CD123^+$ PDCs occurring in reactive LN (data not shown). Interestingly, whereas most if not all PDCs in tonsils and LNs were found to coexpress BDCA-2 and CD123, high endothelial venules (HEVs) were $CD123^+$, but completely negative for BDCA-2 (Fig. 2). Similarly, by immunohistochemical staining of serial sections of normal human thymus, PDCs in thymus were also found to coexpress BDCA-2 and CD123. However, it should be noted that anti–BDCA-2 mAb also stained a considerable proportion of cortical thymocytes, whereas CD123 was totally negative on these cells (data not shown). Whether this is due to some cross-reactivity of anti–BDCA-2 mAb AC144 with molecules on cortical thymocytes or whether positive staining indeed reflects BDCA-2 expression on cortical thymocytes is currently under investigation. Immunohistochemical analysis of testis obtained from patients with seminoma clearly shows that neoplastic and normal epithelium are not stained for BDCA-2, but BDCA-2^+CD123^+ cells displaying a PDC morphology are regularly detectable in association with lymphoid aggregates, that typically occur in this tumor. As commercial first-strand cDNA is not necessarily derived from tissue of healthy donors, this indicates that the presence of BDCA-2 mRNA in the commercial first-strand cDNA from testis (obtained from CLONTECH Laboratories, Inc.) may be attributable to the presence of infiltrating PDCs, rather than attributable to BDCA-2 mRNA expression by normal testis tissue. The same might also be true for pancreas, ovary, and bone marrow.

Identification of Truncated BDCA-2 Transcripts Probably Generated by Alternative Splicing. We detected some size heterogeneity among the PCR-amplified BDCA-2 transcripts. Apart from a band of ~750 bp (full-length BDCA-2 mRNA), at least four additional bands of smaller size were detected (Fig. 4), suggesting the presence of different BDCA-2 mRNA species. This possibility was tested by cloning and sequencing of PCR-amplified BDCA-2 mRNA. At least five truncated BDCA-2 mRNA species that probably represent splice variants were identified (Fig. 4). These are variants lacking (a) exon 3, (b) exon 4, (c) exon 5 (d) exon 3 and 4, and (e) exon 3 and 5. If translated, these putative splice variants would give rise to isoforms of BDCA-2 lacking (a) the transmembrane domain, (b) the neck domain, (c) the whole CRD (lack of exon 5 results in a frame shift which produces a termination codon in exon 6), (d) the transmembrane as well as the neck domain, and (e) the transmembrane as well as the whole CRD.

Apparent Molecular Mass of BDCA-2. To define the biochemical characteristics of BDCA-2, we performed immunoprecipitations from ^{125}I surface-labeled PDCs and

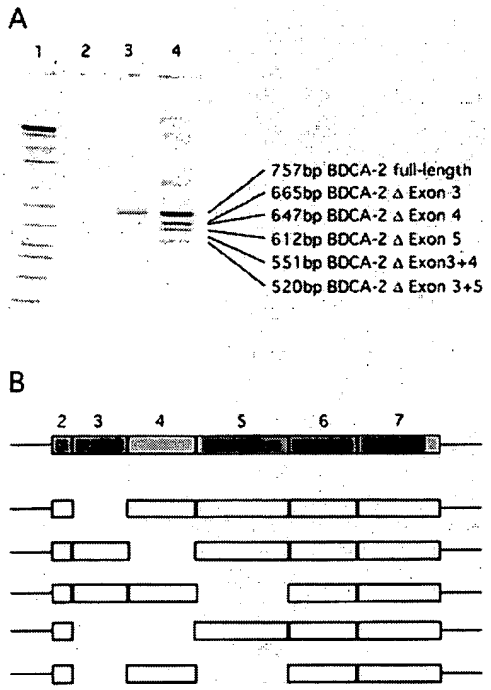

Figure 4. (A) Identification of putative alternative splice forms of BDCA-2 mRNA. Poly(A)$^+$ RNA was isolated from purified PDCs and analyzed for the presence of BDCA-2 mRNA by RT-PCR amplification (lane 2, 20 PCR cycles; lane 3, 25 PCR cycles; lane 4, 30 PCR cycles). The PCR products were size-fractionated by 4–12% TBE PAGE. A further PCR amplification of individual excised bands enabled the cloning and sequencing of individual splice variants. (B) Schematic drawing of the coding region of full-length BDCA-2 mRNA. The structural domains are indicated by different colors (violet, cytoplasmic domain; red, transmembrane domain; green, neck domain; and blue, CRD) and the individual exons (exons 2–7) of the coding region are represented as boxes. Below the full-length BDCA-2 mRNA, BDCA-2 splice variants are shown with the missing exons indicated by gaps.

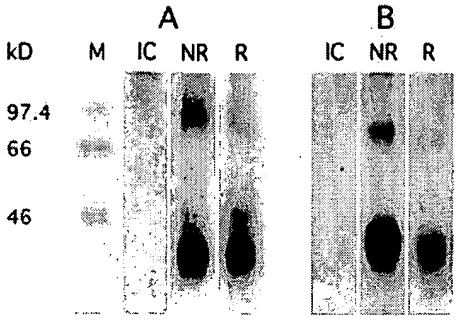

Figure 5. SDS-PAGE analysis of BDCA-2 immunoprecipitated from ^{125}I-labeled PDC (A) and BDCA-2–transfected U937 human monocytoid leukemia cells (B). BDCA-2 appears as a ~38 kD band under nonreducing (NR) and reducing (R) conditions. IC, isotype-matched control mAb. One representative experiment of three is shown.

BDCA-2–transfected U937 cells. A prominent band of ~38 kD was detected in all BDCA-2$^+$ cells under nonreducing and reducing conditions (Fig. 5). The difference between the apparent molecular weight and the molecular mass predicted from the full-length amino acid sequence (25.021 kD), most likely results from N-glycosylation at the N-glycosylation sites (see above). The presence of at least one (PDCs and BDCA-2–transfected U937 cells) or even of two (only PDCs) additional less prominent bands of ~85 kD and ~45 kD, respectively, under nonreducing and reducing conditions, could be due to formation of SDS-stable dimers (85-kD band), differential glycosylation or the presence of coimmunoprecipitated adaptor proteins.

Intracellular Calcium Mobilization after Anti–BDCA-2 mAb Labeling. The possibility that anti–BDCA-2 mAb labeling of PDCs results in signal trunsduction was first addressed by measuring cytosolic calcium concentrations ([Ca^{2+}]i) in PDCs and BDCA-2–transfected U937 and Jurkat cells before and after anti–BDCA-2 mAb labeling. As shown in Fig. 6 B, ligation of surface BDCA-2 with a specific mAb followed by a secondary cross-linking Ab elicited a rapid and transient rise in [Ca^{2+}]i in PDCs and BDCA-2–transfected U937 cells, but not in BDCA-2–transfected Jurkat cells (data not shown) and nontransfected U937 cells. This [Ca^{2+}]i increase was not affected when extracellular calcium was chelated with excess EGTA (Fig. 6 C), indicating that the [Ca^{2+}]i increase is due to intracellular calcium release. Interestingly, the [Ca^{2+}]i increase was much less pronounced when src-family protein-tyrosine kinases were blocked by preincubation with the specific inhibitor PP2 (Fig. 6 D).

Protein-Tyrosine Phosphorylation after Ligation of BDCA-2 with Specific mAb. To test whether intracellular calcium mobilization was paralleled by protein-tyrosine phosphorylation, we performed antiphosphotyrosine immunoblotting on whole cell lysates of purified PDCs, BDCA-2–transfected U937 cells, and BDCA-2–transfected Jurkat cells before and after stimulation with anti–BDCA-2 mAb. As shown in Fig. 7, PDCs and BDCA-2 transfected U937 cells triggered via BDCA-2 displayed a substantial increase in overall protein-tyrosine phosphorylation, as compared with nonstimulated PDCs and BDCA-2–transfected U937 cells, respectively. Like the [Ca^{2+}]i (see above), overall protein-tyrosine phosphorylation is not at all affected by BDCA-2 triggering in BDCA-2–transfected Jurkat cells. This is definitely not due to lack of surface expression of BDCA-2 in Jurkat cells, but may rather be due to lack of an adaptor protein functioning in BDCA-2 signal transduction.

BDCA-2 on PDCs Is Able to Rapidly Internalize and Deliver Anti–BDCA-2 mAb into an Ag-processing Compartment. We have previously shown that labeling of BDCA-2 on PDCs by anti–BDCA-2 mAb and incubation at 37°C induces rapid internalization of the Ag–mAb complex (19). Interestingly, the same is not true for BDCA-2–transfected U937 and Jurkat cells. When BDCA-2–transfected U937 and Jurkat cells were labeled with FITC-conjugated anti BDCA-2 mAb AC144 (IgG1) and incubated for various periods of time at 37°C to allow internalization, the amount of AC144 mAb remaining on the cell surface as determined by staining with PE-conjugated rat anti–mouse

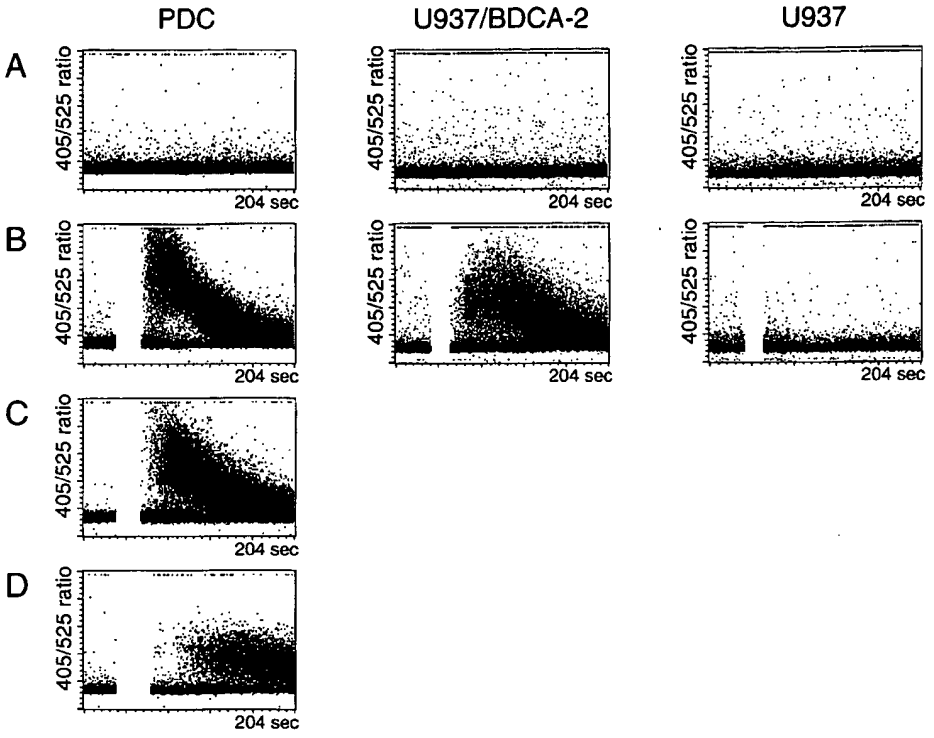

Figure 6. A rapid and transient rise in $[Ca^{2+}]i$ is induced in PDCs (left dotplots) and BDCA-2-transfected U937 cells (middle dotplots), but not in nontransfected U937 cells (right dotplots) after ligation of surface BDCA-2 with specific primary mAb (AC144, IgG1) and secondary cross-linking $F(ab')_2$ goat anti-mouse IgG (B). This $[Ca^{2+}]i$ increase is not affected when extracellular calcium is chelated with excess EGTA (C), but inhibited when src-family protein-tyrosine kinases are blocked by preincubation with the specific inhibitor PP2 (D). One representative experiment of six is shown.

IgG1 mAb did not decrease over time but remained constant (data not shown).

To further investigate a possible role of BDCA-2 on PDCs in Ag capture and Ag presentation, we analyzed the ability of purified PDCs to present AC144 mAb to a $CD4^+$ MHC class II–restricted T cell clone specific for a mouse IgG1-derived peptide epitope (45). As controls, an IgG1 mAb that binds to ILT3 (ZM3.8), a receptor on PDCs known to be capable of delivering its ligand to an intracellular compartment where class II loading occurs (48), and an IgG1 mAb that does not bind to surface molecules on PDCs (anti-cytokeratin mAb CK3–11D5) were used. As shown in Fig. 8, PDCs present AC144 mAb to T cells more efficiently than the anti-ILT3 mAb ZM3.8 and far more efficiently than anti-cytokeratin mAb CK3–11D5. This indicates that BDCA-2 can efficiently deliver its ligand into an Ag-processing compartment.

Ligation of BDCA-2 Inhibits Induction of IFN-α/β Expression in PDCs. PDCs produce large amounts of IFN-α/β when stimulated in vitro with (a) viruses (3–8, 10, 21, 49, 50), (b) bacteria (51) (c) bacterial DNA (oligonucleotides) containing particular unmethylated CpG motifs (CpG-DNA) (14, 22), (d) dsRNA (poly(I:C)) (22), (e) sera from patients with SLE containing an IFN-α/β–inducing factor (most likely complexes of anti-DNA Ab and immunostimulatory hypomethylated DNA (52, 53), or (f) complexes of anti–double-stranded DNA mAb and unmethylated plasmid DNA (human anti-ss/ds DNA mAb MER-3 plus plasmid pcDNA3) mimicking the IFN-α/β–inducing factor present in sera from SLE patients (53). We tested whether in vitro induction of IFN-α/β expression in purified PDCs by FLU, anti-ss/ds DNA mAb MER-3 plus plasmid pcDNA3, and sera from SLE patients is influenced by anti–BDCA-2 mAb labeling of PDCs. As shown in Fig. 9 A, coincubation of purified PDCs with the IFN-α/β–inducing agents and anti–BDCA-2 mAb AC144 (IgG1), but not a nonbinding control IgG1 mAb, dramatically inhibited induction of IFN-α/β expression as compared with incuba-

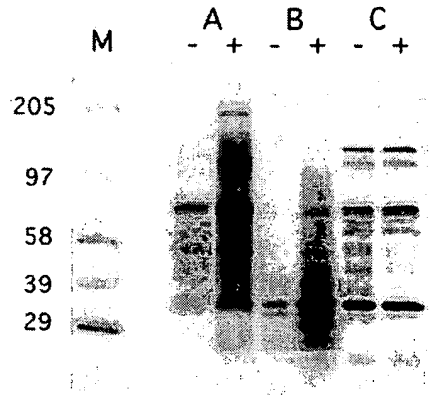

Figure 7. Triggering of BDCA-2 induces protein tyrosine phosphorylation in purified PDCs (A) and BDCA-2 transfected U937 cells (B), but not in BDCA-2–transfected Jurkat cells (C). Cells were incubated with medium alone (−) or with anti–BDCA-2 mAb (AC144, IgG1) (+). Cell lysates were size-fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with horseradish peroxidase-coupled antiphosphotyrosine mAb PY20. One representative experiment of two is shown.

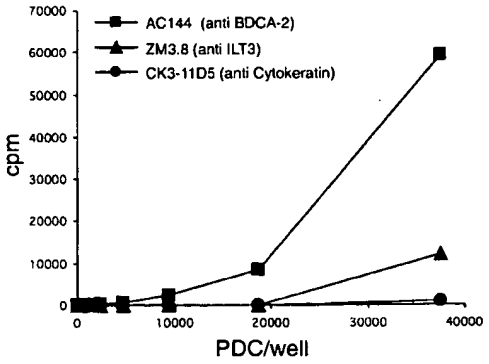

Figure 8. Presentation of anti-BDCA-2 mAb (AC144, IgG1) to a T cell clone specific for mouse IgG1 by irradiated PDCs. Anti-BDCA-2 mAb AC144 (■) is presented more efficiently than anti-ILT3 mAb ZM3.8 (▲) and far more efficiently than anti-cytokeratin mAb CK3-11D5 (●). One representative experiment of two is shown.

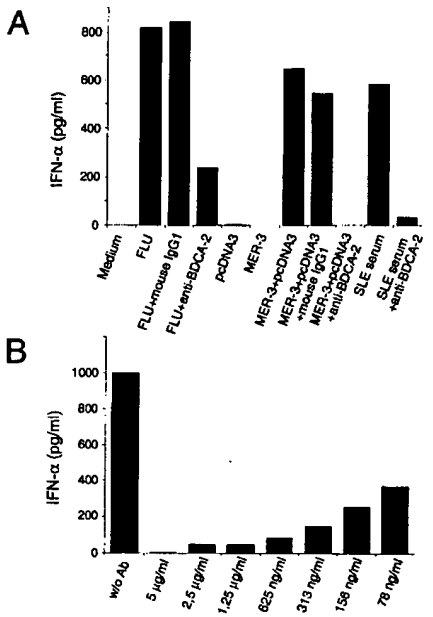

Figure 9. (A) Ligation of BDCA-2 suppresses induction of IFN-α/β production in PDCs. Stimulation of PDCs with FLU, anti-ss/ds DNA mAb MER-3 plus plasmid pcDNA3, or serum from a SLE patient, but not with anti-ss/ds DNA mAb MER-3 alone or plasmid pcDNA3 alone, induces production of large amounts of IFN-α/β in PDCs. Induction of IFN-α/β production with this agents can be inhibited by coincubation with anti-BDCA-2 mAb (AC144, IgG1), but not by coincubation with an isotype-matched control IgG1 mAb. The data shown are representative of more than six experiments using FLU, more than two experiments using anti-ss/ds DNA mAb MER-3 plus plasmid pcDNA3, and three experiments using SLE sera as IFN-α/β production–inducing agent. (B) Anti–BDCA-2 mAb–mediated suppression of induction of IFN-α/β production is mAb concentration dependent. Purified PDCs were coincubated with one of the IFN-α/β–inducing agents (anti-ss/ds DNA mAb MER-3 plus plasmid pcDNA3) and titrated amounts of anti–BDCA-2 mAb (AC144, IgG1). Note that concentrations below 100 ng/ml are sufficient for a 50% inhibition of the IFN-α/β response.

tion of PDCs with the IFN-α/β–inducing agents alone. Similar results were obtained using CpG oligodeoxynucleotides (AAC-30 and GAC-30) and a killed streptococcal preparation (OK432) for induction of IFN-α/β expression in PDCs (data not shown). Interestingly, with most IFN-α/β–inducing agents (e.g., anti ss/ds DNA mAb MER-3 plus plasmid pcDNA3 or sera from SLE patients), we reproducibly obtained complete or nearly complete blocking of induction of IFN-α/β expression by anti–BDCA-2 mAb, whereas only partial blocking was obtained using FLU as IFN-α/β–inducing agent. Titration of the anti–BDCA-2 mAb (Fig. 9 B) reveals that concentrations below 100 ng/ml are sufficient for a 50% inhibition of the IFN-α/β response to anti-ss/ds DNA mAb MER-3 plus plasmid pcDNA3. To check whether anti–BDCA-2 mAb inhibits induction of IFN-α/β expression simply by PDC killing or by induction of PDC maturation, we comparatively analyzed total numbers of viable PDCs and expression of several leukocyte Ag (CD83, CD80, CD86, etc.) after various periods of culturing of PDCs in the presence or absence of anti–BDCA-2 mAb. No significant differences were found (data not shown).

Discussion

DCs constitute a heterogeneous population of cells with different developmental pathways and maturation stages. While several leukocyte surface Ag, including costimulatory molecules, chemokine receptors, endocytic/phagocytic receptors, and typical DC activation/maturation Ag are differentially regulated during DC maturation (54), their expression is not restricted to DCs. Surface Ag restricted to DC subsets, such as PDCs, however, are highly valuable tools for cell monitoring in healthy volunteers and patients. We have previously demonstrated that BDCA-2 is an excellent marker for the identification of PDCs in blood (19). The present study extends this finding to lymphoid (LNs, tonsils, thymus) and nonlymphoid (testis) tissue. In line with the Ab staining data, our RT-PCR analysis also indicates a strict restriction of BDCA-2 mRNA expression to PDCs.

DCs are found to express a huge number of lectins that appear to serve quite distinct functions. Members of the C-type lectin family can be divided into two groups based on their molecular structures: (a) type I surface lectins with multiple CRD in the NH_2-terminal extracellular region, and (b) type II surface lectins with a single CRD in the COOH-terminal extracellular region. DC express type I as well as type II lectins. Endocytic activity, a hallmark of receptor-mediated uptake of Ag, is exhibited by type I multi-CRD lectins, such as the macrophage-mannose receptor (MMR; reference 55) and DEC-205 (56), as well as by type II single-CRD lectins, such as CD23 (57) and Langerin (58). Langerin is specifically expressed by Langerhans cells, whereas the MMR, CD23, and DEC-205 are not specifically expressed by DCs in general or by a particular DC subset. Our data indicate that BDCA-2 is a unique endocytic type II single-CRD lectin specifically expressed in

PDCs and able to target ligand into Ag processing and peptide-loading compartments for presentation to T cells.

In addition to its Ag-capturing function, BDCA-2 can mediate potent inhibition of induction of IFN-α/β expression in PDCs. Production of IFN-α/β in response to several different types of viruses (3–8, 10, 21, 50), bacteria (51), CpG-DNA (14, 22), dsRNA (22), and SLE serum (52, 53) is by far the most prominent feature of PDCs. It has recently been shown that PDCs express high amounts of toll-like receptor 9 (TLR9; reference 59) and that TLR9 confers responsiveness to CpG-DNA (60). Thus, TLR9 is a prime candidate for mediating induction of IFN-α/β expression in PDCs, but, in the light of the wide variety of IFN-α/β–inducing agents, other receptors are likely to serve the same function. Our data show that triggering of BDCA-2 can potently inhibit in vitro induction of IFN-α/β expression in PDCs by (a) viruses, (b) the combination of anti-dsDNA mAb and unmethylated plasmid DNA, (c) SLE serum, (d) CpG oligonucleotides, and (e) OK432. The diversity of IFN-α/β–inducing agents makes it anyway highly unlikely that anti–BDCA-2 mAb AC144 simply blocks binding of the IFN-α/β–inducing agents to PDCs, but this can also be ruled out by the fact that induction of IFN-α/β expression is also suppressed if the IFN-α/β–inducing agent is added 1 h before the anti–BDCA-2 mAb (data not shown).

SLE patients have increased levels of IFN-α/β in the circulation and increased production of IFN-α/β may well be of pathogenic significance in SLE (61–63). This is strongly supported by the observation that IFN-α/β therapy of patients with nonautoimmune diseases occasionally induces the development of Ab against dsDNA and SLE-like syndromes (64-67). PDCs have been proposed to be a major source of IFN-α/β in SLE (52), but the observation that SLE patients have a reduced number of PDCs in peripheral blood (68) seemed to contradict this hypothesis. However, recent studies by Farkas et al. (35) and Blomberg et al. (34) have demonstrated accumulation of PDCs in cutaneous SLE lesions. This indicates that the low numbers of PDC in blood are due to their increased recruitment to tissues. Furthermore, by showing that the density of PDCs in affected skin correlated well with the number of cells expressing the IFN-α/β–inducible protein MxA, Farkas et al. (35) provided strong evidence that PDCs are indeed a major source of IFN-α/β in SLE. Based on our in vitro results it appears reasonable to evaluate a novel strategy for inhibiting IFN-α/β production by PDCs in SLE patients, namely by means of in vivo administration of anti–BDCA-2 mAb.

BDCA-2 showed the highest degree of homology to murine dectin-2 (50.7% amino acid identity in the overall sequence; reference 41) and to human DCIR (42.1% amino acid identity in the overall sequence; reference 42). BDCA-2 and dectin-2 differ from DCIR in that the immunoreceptor tyrosine-based inhibitory motif (ITIM) found in the intracellular domain of DCIR was absent from the relatively short intracellular domains of dectin-2 (14 amino acids) and BDCA-2 (21 amino acids). The intracellular domains of BDCA-2 and dectin-2 also lack an immunoreceptor tyrosine based activation motif (ITAM) and any other known motif implicated in signal transduction. Based upon these observations, Ariizumi et al. (41) concluded that dectin-2 has no signaling potential. We found that triggering of BDCA-2 induces src-family protein-tyrosine kinase–dependent intracellular calcium mobilization and protein-tyrosine phosphorylation of intracellular proteins. Thus, though no apparent signaling motif is present in the intracellular domain of BDCA-2, BDCA-2 clearly is a signaling receptor that activates protein-tyrosine kinases. Previous studies on the regulation of IFN-α/β production in PDCs indicate that activation of protein kinases may play a direct role in the BDCA-2–mediated inhibition of IFN-α/β induction (69, 70). It remains to be analyzed whether signaling is accomplished by the pairing of BDCA-2 with a signaling membrane adaptor molecule, such as DAP12. DAP12 associates noncovalently with a large number of receptors in natural killer and myeloid cells, including several membrane glycoproteins of the C-type lectin superfamily. All associations are based on the presence of a negatively charged residue in the transmembrane domain of DAP12 and a positive charge in the transmembrane domain of the receptor (71, 72). As positively charged amino acid residues are not present in the transmembrane domain of BDCA-2, it is rather unlikely that DAP12 itself associates with BDCA-2.

Unfortunately, no information is currently available on the natural ligands and the carbohydrate specificities of DCIR, dectin-2, and BDCA-2. In fact, a His-tagged recombinant protein containing the extracellular domain of dectin-2 showed no specific binding to any of a panel of carbohydrate probes (41). Future studies are required to identify the ligand(s) of BDCA-2.

In this context, it may also be worth mentioning that at least five different truncated BDCA-2 transcripts, which are most likely generated by alternative splicing, have been identified. If these truncated BDCA-2 transcripts are translated, all of them completely lack one or two structural components: the transmembrane domain alone, the neck domain alone, the CRD alone, the transmembrane domain and the neck domain, or the transmembrane domain and the CRD. Two truncated isoforms have also been identified for dectin-2, one with a 34 amino acids deletion in the neck domain and one with a 41 amino acids deletion within the CRD (41). Isoforms lacking the transmembrane domain might serve as soluble receptors controlling membrane-bound receptor-mediated functions.

In conclusion, in this study, we report the molecular cloning and functional characterization of BDCA-2, a novel lectin specifically expressed by PDCs. Remarkably, BDCA-2 is a PDC-specific transmembrane lectin that appears to display a dual function of Ag-capturing and potent inhibition of IFN-α/β induction. As production of IFN-α/β by PDCs has been implicated to play an important role in innate antiviral immunity (21) and Th1 polarization (20), triggering of BDCA-2 may be critical for skewing T cell responses toward a non-Th1 type of response and enable viruses to escape from the host immune defense.

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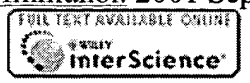

Molecular and genomic characterization of human DLEC, a novel member of the C-type lectin receptor gene family preferentially expressed on monocyte-derived dendritic cells.

Arce I, Roda-Navarro P, Montoya MC, Hernanz-Falcon P, Puig-Kroger A, Fernandez-Ruiz E.

Unidad de Biologia Molecular, Hospital Universitario de la Princesa, Madrid, Spain.

We have identified a novel gene encoding a protein designated DLEC (dendritic cell lectin), which is a type II membrane glycoprotein of 213 amino acids and belongs to the human calcium-dependent (C-type) lectin family. The cytoplasmic tail of DLEC lacks consensus signaling motifs and its extracellular region shows a single carbohydrate recognition domain (CRD), closest in homology to the dendritic cell immunoreceptor (DCIR) CRD. The DLEC gene has been localized linked to DCIR on the telomeric region of the NK gene complex. RT-PCR and Northern blot analyses show that DLEC mRNA is preferentially expressed in monocyte-derived dendritic cells.

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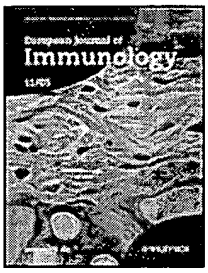

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Molecular and genomic characterization of human *DLEC*, a novel member of the C-type lectin receptor gene family preferentially expressed on monocyte-derived dendritic cells

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We would like to dedicate this work to the late Dr. J. Tormo.

Keywords

C-type lectin gene; NK gene complex; Dendritic cell

Abstract

We have identified a novel gene encoding a protein designated DLEC (dendritic cell lectin), which is a type II membrane glycoprotein of 213 amino acids and belongs to the human calcium-dependent (C-type) lectin family. The cytoplasmic tail of DLEC lacks consensus signaling motifs and its extracellular region shows a single carbohydrate recognition domain (CRD), closest in homology to thedendritic cell immunoreceptor (DCIR) CRD. The *DLEC* gene has been localized linked to *DCIR* on the telomeric region of the NK gene complex. RT-PCR and Northern blot analyses show that *DLEC* mRNA is preferentially expressed in monocyte-derived dendritic cells.

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